Serine Palmitoyltransferase and Peripheral Neuropathy - Studies on Neuropathy-causing Mutations and their Biochemical Hallmarks

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Heiko Sebastian Bode

aus

Deutschland

Promotionskomitee

Prof. Dr. Arnold von Eckardstein (Vorsitz, Leitung der Dissertation)
Prof. Dr. Thorsten Hornemann
Prof. Dr. Esther Stoeckli
Dr. Omolara Ogunshola

Zürich, 2016

To Paulina, the smallest, biggest, wonder of my life,

and her not less wonderful mother Julia.

Declaration of Originality

I, Heiko Bode, hereby declare that this thesis represents my original work and that I have used no other sources except the ones indicated by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

Neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

Heiko Bode

Urdorf, 22.12.2015

Abstract

The multimeric enzyme serine palmitoyltransferase (SPT) catalyzes the first step of the sphingolipid (SL) *de novo* synthesis, the condensation of palmitoyl-CoA and L-serine to the first sphingoid base sphinganine. Missense mutations in two of three SPT subunits (serine palmitoyltransferase long chain base subunit 1 and 2, SPTLC1 and SPTLC2) cause the rare hereditary sensory and autonomic neuropathy type 1 (HSAN1). HSAN1 is a slowly progressive sensory neuropathy with an onset in adulthood, usually between 2nd and 5th decade. Several disease-causing mutations of SPT increase the promiscuous activity of SPT with the alternative substrates L-alanine and glycine which results in increased formation of neurotoxic 1-deoxysphingolipids (1-deoxySLs). Due to a missing C1 hydroxyl group, 1-deoxySLs can neither be converted to complex sphingolipids nor degraded by the classical pathway. Elevated 1-deoxySL concentrations were consistently found in nerves and plasma of the HSAN1 mouse model and in plasma and lymphoblasts of HSAN1 patients. Increased concentrations of 1-deoxydSLs in plasma represent a reliable biomarker for the diagnosis of SPT-associated HSAN1. Today thirteen different missense mutations of SPTLC1 and SPTLC2 have been conclusively associated with HSAN1. Besides this genotypical variability the phenotypes are also quite diverse.

Several cases with atypical severe symptoms starting in early childhood and even congenital have been reported. Here we report, the case of a patient with a new mutation of serine 331 in SPTLC1 (exchange of serine for tyrosine; p.S331Y). The patient presents severe and atypical symptoms such as: early onset, growth retardation, ocular manifestations and respiratory problems. This phenotype is very similar to the symptoms, caused by another mutation at the same residue (p.S331F). Serine 331 obviously is a crucial residue for SPTLC1 and mutations of this residue cause a distinct and severe syndrome.

Mutations of S331 are rare. Only four patients with mutations of serine 331 have been reported so far and one of them had died at the age of 28years already. In all four cases the mutation did not segregate but had occurred *de novo*. Our previous work had shown that formation and accumulation of 1-deoxySLs, by mutant-expressing HEK cells, can be effectively prevented by L-serine supplemented medium. We measured significantly reduced 1-deoxySL concentrations, as a result of a dietary L-serine supplementation, in plasma and tissues of the mouse model and also in plasma from HSAN1 patients with the p.C133Y mutation. These promising results raised the hope, that L-serine supplementation might be the first long-term therapy for all SPT-associated HSAN1.

We launched and accompanied an experimental L-serine trial with one of the three known S331F patients, to test, whether this therapeutic approach could be used even for this severe

case. Repeated measurements of the sphingolipid profile of this patient over two years confirmed our hypothesis. Concentrations of 1-deoxySLs were reduced to 45% of the baseline levels and the patient reported significant improvements in temperature sensation, wound healing, muscle strength, endurance, hair growth and body weight. Once more, this proves the big potential of dietary L-serine supplementation as standard treatment for SPT-associated HSAN1 in general.

Genotypic variability of HSAN1 is high, and several new mutations of SPT are being discovered almost every year. Another missense mutation within SPTLC1 (alanine to valine exchange at position 339, p.A339V) was recently found in a German family. Several members of this family presented mild symptoms of a peripheral neuropathy. We analyzed the sphingolipid profiles of affected and healthy members of this family and found increased concentrations of 1-deoxySLs in all affected individuals. This finding conflicts with the genotypes, as two of the neuropathy patients do not have the mutation and a female carrier of the mutation does not present any symptoms of the disease. A causative role of A339V alone therefore can be excluded. This raises the question, how the 1-deoxySLs in the non-carriers were formed without the involvement of a mutation in SPT.

To better understand the underlying mechanisms of 1-deoxySL formation and the differences between the mutations, we compared the biochemical properties of all known mutations in SPTLC1 and SPTLC2. We used mutant-overexpressing HEK293 cells, metabolic labeling and LC-MS to analyze activity and substrate preference of mutant SPT in different conditions. The canonical activity of SPT was not reduced in in any of the mutants and three of them even caused hyperactivity. Eight mutants had significant higher activity with alanine. With the generated dataset, we were able to group the mutations into distinct clusters based on their biochemical properties. This biochemical clustering of the mutants is reflected by the severity of the phenotypes and the sphingolipid profiles in plasma of the affected patents. The formation of 1-deoxySLs is a crucial factor for the neuropathy and the activity of SPT with L-alanine correlates with the severity of the disease. Canonical hyperactivity worsens the effect of 1-deoxySL formation and causes the most severe pathologies.

This thesis highlights the genotypic and phenotypical variability of HSAN1. Our biochemical comparison shows common characteristics of all SPT mutations and provides a comprehensive set of simple experimental methods to determine them. The genotype-phenotype correlation allows forecasts on the course and severity of the disease directly after diagnose. We showed that L-serine supplementation is a promising long term therapy even for the rare and severe cases of HSAN1. We found further strong hints that mutations in SPTLC1 and SPTLC2 might not be the only source for increased 1-deoxySLs in HSAN1

patients. The mechanism of 1-deoxySL formation and regulation of SPT activity is very complex and more work will be necessary to finally unravel its enigmatic nature.

Zusammenfassung

Das multimere Enzym Serin palmitoyltransferase (SPT) katalysiert den ersten Schritt der Sphingolipid *de novo* Synthese, die Kondensation von L-Serin und Palmitoyl-CoA zur ersten Sphingoid Base Sphinganin. Missense-Mutationen in zwei der drei SPT Untereinheiten (Serine palmitoyltransferase long-chain base subunit 1 und 2, SPTLC1 und SPTLC2) verursachen die seltene Hereditäre sensorisch-autonome Neuropathie Typ 1 (HSAN1). HSAN1 ist eine langsam voranschreitende sensorische Neuropathie, die für gewöhnlich erst im Erwachsenenalter zwischen dem 2ten und 5ten Lebensjahrzehnt beginnt. Mehrere krankheitsverursachende Mutationen der SPT steigern die die promiskuitive Aktivität der SPT mit den alternativen Substraten L-Alanin und Glycin, was zu einer gesteigerten Bildung von neurotoxischen 1-Deoxysphingolipiden (1-deoxySL) führt. Aufgrund der fehlenden C1 Hydroxylgruppe, können die 1-deoxySL weder zu komplexen Sphingolipiden umgeandelt, noch über den klassischen Weg abgebaut werden. Erhöhte Konzentrationen von 1-Deoxysphingolipiden wurden immer wieder in Nerven und Plasma des HSAN1 Maus-Modells sowie im Plasma und in Lymphoblasten von HSAN1 Patienten gefunden. Gesteigerte 1-deoxySL-Konzentrationen im Plasma stellen einen zuverlässigen Biomarker für die Diagnose der SPT-verursachten HSAN1 dar. Bis heute wurden dreizehn verschiedene Missense-Mutationen in SPTLC1 und SPTLC2 eindeutig mit HSAN1 assoziiert. Neben dieser genetischen Variabilität sind auch die Phänotypen recht unterschiedlich. Es wurden bereits mehrere Fälle mit aussergewöhnlich schwerwiegenden Symptomen, die auch bereits in der frühen Kindheit oder sogar mit der Geburt einsetzten, gemeldet.

Hier schildern wir den Fall einer Patientin mit einer neuen Mutation von Serin 331 in SPTLC1 (Austausch von Serin gegen Tyrosin, p.S331Y). Die Patientin zeigte schwerwiegende und untypische Symptome wie etwa: frühen Beginn, Wachstumsverzögerung, okuläre Manifestationen und Beeinträchtigung der Atmung. Dieser Phänotyp ähnelt damit sehr den Symptomen, die durch eine weitere Mutation derselben Aminosäure (p.S331F) hervorgerufen werden. Serin 331 ist offensichtlich eine essentiell wichtige Aminosäure für SPTLC1 und Mutationen dieser Aminosäure verursachen ein klar abgegrenztes und schwerwiegendes Syndrom.

Mutationen von S331 sind selten. Bisher wurden lediglich vier Patienten mit Mutationen von Serin 331 berichtet und einer von diesen vier ist bereits im Alter von 28 Jahren verstorben. Die Mutation wurde in allen vier Fällen nicht etwa vererbt, sondern war jeweils neu aufgetreten. Unsere bisherigen Arbeiten hatten bereits gezeigt, dass die Bildung und Akkumulation von 1-Deoxysphingolipiden in HEK-Zellen, welche die Mutationen exprimieren, durch Zugabe von L-Serin zum Medium wirkungsvoll verhindert werden kann. Als Folge

einer Nahrungsergänzung mit L-Serin, konnten wir bereits, sowohl in den Nerven und im Plasma des Maus Modells, als auch in HSAN1 Patienten mit der p.C133Y Mutation, deutlich verringerte 1-deoxySL-Konzentrationen messen. Diese vielversprechenden Ergebnisse erweckten die Hoffnung, dass die L-Serin Supplementierung die erste Langzeittherapie für alle SPT-verursachten Fälle von HSAN1 sein könnte.

Um zu überprüfen, ob dieser therapeutische Ansatz auch für diesen schwerwiegenderen Fall verwendet werden kann, haben wir einen L-Serin Versuch mit einem der drei p.S331F-Patienten gestartet und begleitet. Wiederholte Analysen des Sphingolipidprofils dieses Patienten, über einen Zeitraum von zwei Jahren, bestätigten unsere Hypothese. Die 1-deoxySL-Konzentration im Plasma des Patienten ging bis auf 45% des Ausgangswertes zurück und der Patient berichtete deutliche Verbesserungen seiner Temperaturwahrnehmung, seiner Wundheilung, seiner Muskelkraft und Ausdauer, seines Haarwuchses und seines Körpergewichtes. Dies beweist einmal mehr das grosse Potential der L-Serin Supplementation als Standardbehandlung für alle SPT-verursachten Fälle von HSAN1.

Die genetische Variabilität von HSAN1 ist hoch und neue Mutanten der SPT werden fast jedes Jahr entdeckt und gemeldet. Eine weitere Missense-Mutation in SPTLC1 (ein Austausch von Alanin durch Valin an Position 339, p.A339V) wurde kürzlich in einer deutschen Familie entdeckt. Mehrere Familienmitglieder litten unter milden Symptomen einer peripheren Neuropathie. Wir haben die Sphingolipidprofile von betroffenen und gesunden Familienmitgliedern analysiert und dabei erhöhte 1-deoxySL-Konzentrationen in allen betroffenen Individuen gefunden. Diese Entdeckung widerspricht den Genotypen, da zwei der Neuropathiepatienten die Mutation nicht in ihrem Genom tragen und eine weibliche Trägerin der Mutation keinerlei Krankheitssymptome aufweist. Es kann daher ausgeschlossen werden, dass p.A339V alleine die Symptome verursacht. Dies wiederum wirft die Frage auf, wie die 1-deoxySL in den Nicht-Trägern ohne die Beteiligung einer SPT-Mutation gebildet wurden.

Um den zugrunde liegenden Mechanismus der 1-deoxySL-Bildung und die Unterschiede zwischen den verschiedenen Mutationen besser zu verstehen, haben wir die biochemischen Eigenschaften von allen bekannten Mutationen der SPTLC1 und SPTLC2 verglichen. Um die Aktivität und Substratpräferenz der SPT unter verschiedenen Bedingungen zu messen, haben wir HEK293-Zellen verwendet, die die SPT-Mutanten überexprimieren. Nach metabolischer Markierung wurden die gebildeten Sphingolipide mittels LC-MS analysiert. Die kanonische Aktivität der SPT war in keiner der untersuchten Mutanten reduziert und drei Mutationen verursachten sogar Hyperaktivität. Acht Mutanten zeigten eine gesteigerte Aktivität mit Alanin. Mit dem erzeugten Datensatz konnten wir die Mutationen aufgrund ihrer

biochemischen Eigenschaften unterschiedlichen Clustern zuordnen. Diese biochemische Zuordnung der Mutanten spiegelt sich auch im Schweregrad der verursachten Phänotypen sowie den Sphingolipidprofilen im Plasma der Patienten wieder. Die Bildung von 1-deoxySL ist ein entscheidender Faktor für die Neuropathie und die Aktivität der SPT mit L-Alanin korreliert mit der Schwere der Erkrankung. Kanonische Hyperaktivität verschlimmert die Wirkung der 1-deoxySL-Bildung zusätzlich und verursacht so letztlich die schwerwiegendsten Pathologien.

Diese Arbeit unterstreicht die genetische und phänotypische Variabilität der HSAN1. Unser Biochemischer Vergleich zeigt die Gemeinsamkeiten aller bekannter SPT Mutationen auf und liefert ein umfassendes Set von einfachen, experimentellen Methoden, um diese zu bestimmen. Die Genotyp-Phänotyp-Korrelation erlaubt die Vorhersage des Verlaufs sowie des Schweregrades der zu erwartenden Symptome direkt nach der Diagnose. Wir haben gezeigt, dass L-Serin Supplementierung selbst für die seltenen, schwerwiegenden Fälle von HSAN1 eine vielversprechende Langzeittherapie darstellt. Wir haben darüber hinaus weitere Hinweise dafür gefunden, dass Mutationen in SPTLC1 und SPTLC2 möglicherweise nicht die einzigen Ursachen für erhöhte 1-deoxySL-Werte sind. Der Mechanismus der 1-deoxySL-Bildung und Regulation der SPT ist sehr komplex und mehr Arbeit wird notwendig sein, um ihre Rätselhaftigkeit endgültig zu entschlüsseln.

Table of Contents

1 General Introduction

1.1 Peripheral neuropathies

Peripheral neuropathies are neurologic disorders affecting nerves outside of the central nervous system (brain and spinal cord). Impaired signal transmission within the affected nerves causes deficits such as impaired gland and organ function, but also impairments of temperature and pain perception, mechanosensation, proprioception, muscular coordination and maintenance of autonomic functions. Symptoms strongly depend on the affected nerves and the type of neuropathy.

Peripheral neuropathies are frequent neurologic complications. With a total prevalence of 2-8% they are at least as common as stroke (1).

Frequently, neuropathic complications are associated with metabolic or systemic diseases and infections such as type 2 diabetes mellitus (T2DM) or leprosy, but they can also arise from other sources like vitamin B_{12} deficiency, chemotherapy, alcohol abuse, heavy metal intoxications, traumatic mechanical injuries of the respective nerve, and immune system disorders (2).

Based on the predominantly affected structures, peripheral neuropathies can be subdivided into demyelinating and axonal neuropathies. Damage of Schwann cells causes segmental demyelination of the axonal fibers which typically results in a significant reduction of nerve conduction velocities in the affected nerves. The Guillain-Barré syndrome, diphteric neuropathy and chronic inflammatory demyelinating polyneuropathy (CIDP) are typical diseases associated with demyelination of axonal fibers. Recovery from this condition however is possible and can occur quite fast by remyelination of the affected nerves (2, 3). Demyelinating neuropathies can cause a length-dependent pattern of sensory impairments, as the longest fibers also have the highest probability of becoming demyelinated and blocked. Widespread reflex loss in muscle groups that are not weak or wasted is a hallmark feature typically only associated with demyelination (1).

The most conspicuous neuropathic symptoms however are caused by the direct impairment of axons leading to a dying back phenotype and retraction of axonal fibers from the periphery. Recovery from axonal degradation is slow and often incomplete (3). Dying back axonal neuropathies typically present a length dependent and symmetrical pattern of

symptom evolution. Motor involvement, weakness and muscle wasting most frequently start in the distal limbs and slowly spread proximally. Sensory loss is usually noticed in a glove and stocking pattern (see Figure 1-1 A), progresses proximally, and can eventually reach the midline of the abdomen (see Figure 1-1 B). Selective loss of ankle jerks together with distal muscle wasting or weakness is a hallmark of distal axonal neuropathies (1).

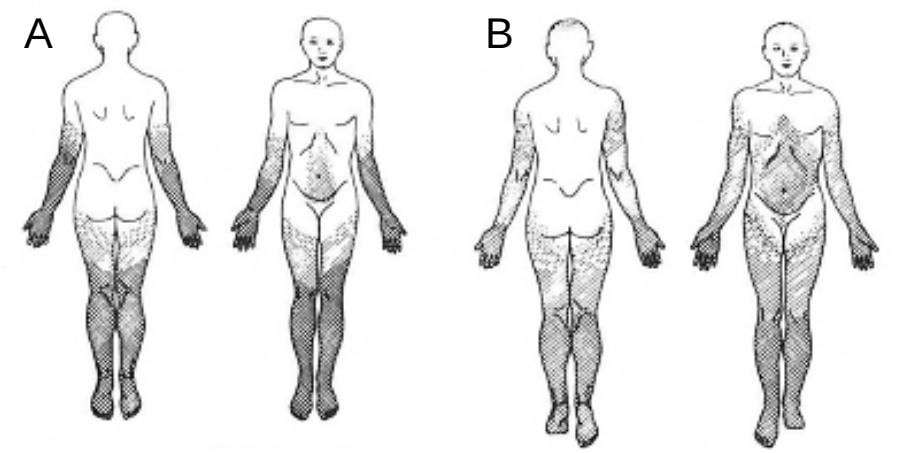


Figure 1-1 Distribution of sensory loss/impairment in diabetic sensory polyneuropathy (showcase for distal symmetric polyneuropathies).
A; At an early stage, pinprick response is lost in distal lower and upper limbs (dark shaded areas) and impaired in the more proximal parts of the extremities above knee and elbow, and in a small strip around the navel (light shaded areas)
B; With progression of the disease, sensory loss aggravates, spreads proximally, and affects large areas of the trunk, as well as the vertex of the head. Figure taken from: (2)

1.2 Inherited peripheral neuropathies

About 50-70% of all patients with a peripheral neuropathy carry an undiagnosed inherited cause for it (4, 5). Inherited neuropathies form a large and heterogeneous group of diseases that are primarily affecting the peripheral nerves. They can be further discriminated into two groups: one group in which the neuropathy is the only or predominant symptom of the disease; and the other group in which it is only one symptom out of a more complex neurological or even multisystemic disorder (6).

Diseases like Charcot-Marie-Tooth (CMT), hereditary sensory and autonomic neuropathy (HSAN), distal hereditary motor neuropathies (DHMN), hereditary neuralgic amyotrophy and familial amyloid polyneuropathy (FAP) are summarized within the first group, while disturbances of lipid metabolism like the sphingomyelin lipidosis (Niemann-Pick disease), porphyrias, and hereditary ataxias (e.g. Friedreich´s ataxia) are some examples belonging to the second group (6).

1.3 CMT:

Charcot-Marie-Tooth (CMT) and related disorders form the largest group of inherited neuropathies, with a marked genetic and clinical variability. A duplication in the short arm of chromosome 17 (17p), which contains the gene for peripheral myelin protein 22 (PMP22) is the first and most common genetic cause of CMT and causes the phenotype CMT1A. The 17p duplications together with some other mutations in GJB1, PMP22 and MPZ account for 76% of all cases of CMT1 and 42% of all cases of CMT. (7).

The classical CMT phenotype is characterized by length-dependent, slowly progressing distal muscle wasting and weakness starting in the lower limbs, reduced tendon reflexes, variable impairment of distal sensation, variable foot deformities, and motor and sensory neuropathic impairments (6). The estimated overall prevalence of inherited CMT is 1 in 2500, which makes it one of the most common groups of neuropathies (7, 8).

The introduction of modern neurophysiological techniques allowed regrouping of CMT and CMT-related diseases according to their mode of impairment of nerves (see Figure 1-2).

The demyelinating forms of CMT (as caused by duplication of 17p) are defined by clearly reduced motor conduction velocities (MCV) << 38m/s and typically cause the CMT1 phenotype. Only moderate reductions of MCV > 38m/s) are presented by the axonal forms causing the CMT2 phenotype. Mutations in GJB1 are typically associated with MCV between 25 and 45 m/s and cause various intermediate phenotypes (7).

The axonal forms of CMT-related diseases comprise three subgroups of phenotypically similar axonopathies (see Figure 1-2). The first group, CMT2, is characterized by pronounced motor and sensory involvement. In the second group, hereditary sensory neuropathies (HSN), the sensory and autonomic impairments are usually dominant and less motor impairment is observed, while in the third group, the distal hereditary motor neuropathies (dHMN), motoric defects clearly dominate the clinical presentation (6).

Today mutations in more than 80 genes have been associated with CMT-related phenotypes. Most of them are known to cause only one specific phenotype, but some genes have been associated with various phenotypes and even different modes of inheritance. Mutations in HSPB1 for example are associated with CMT2 and distal HMN and can be inherited either in an autosomal dominant or recessive manner.

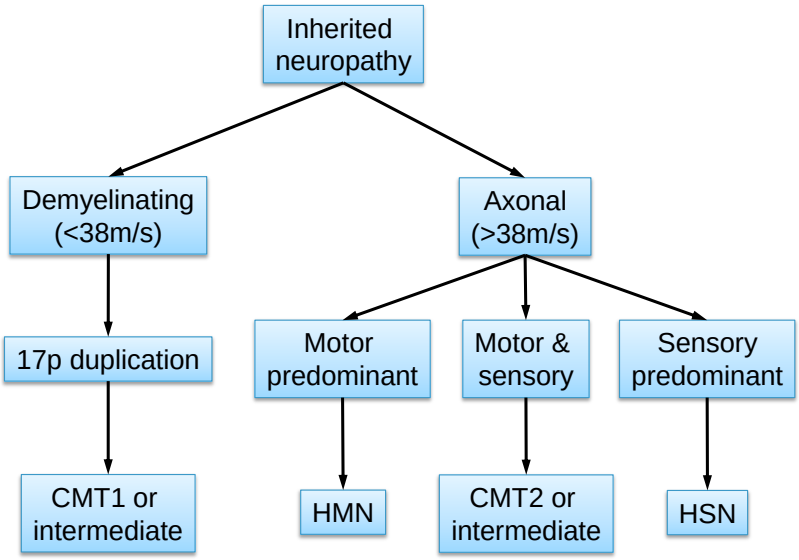


Figure 1-2 simplified diagnostic guideline for CMT and related disorders adapted from (7)

1.4 Axonal neuropathies CMT and HSN

Phenotypical discrimination between the three types of axonal neuropathies (see Figure 1-2) and their subtypes is based on the distribution and type of the presented symptoms (motor impairments versus sensory loss). Typical diagnostic hallmark symptoms include foot deformities, stepper gait, ulcer formation, and the age of onset.

Motor nerve involvement is highly variable amongst the different HSN subtypes. Characteristic clinical symptoms like pronounced sensory impairments and ulcerations predominantly forming at the feet can be the only factors to discriminate hereditary sensory and autonomic neuropathy type 1 (HSAN1) from CMT, for example (9). Genotypic discrimination does not necessarily increase diagnostic precision, as mutations in the small GTPase RAB7 for example are associated with the allelic phenotypes CMT2B and HSAN1, and mutations in HSPB1 can cause CMT2F and HMN2B (7).

1.4.1 HSAN1

Hereditary sensory and autonomic neuropathy type 1 (HSAN1) is a slowly progressive dominantly inherited peripheral axonal neuropathy (9) and encompasses several subtypes of peripheral neuropathies with prominent sensory and variable autonomic involvement.

Literature analyzes of Dyck and Houlden (10, 11), suggest that the first cases of HSAN1 were reported in early French literature around 1850. In 1846, Leplat described an individual case with symptoms of an ulcerating neuropathy (12) and Nelaton reported the inheritability of this disease in a kinship group with three out of six brothers being affected by a peripheral neuropathy (13). In 1922 Hicks reported the case of a large British family with a remarkable 10 out of 34 family members presenting the cardinal symptoms such as perforating ulcers predominantly affecting the lower limbs, shooting pains about the body, which resemble *tabes dorsalis*, and progressive deafness. He therefore initially assigned the name "hereditary perforating ulcer of the feet" to the disease (14). Thirty years later Denny-Brown conducted an autopsy of one female patient out of the kinship reported by Hicks. The examination of the patient's central and peripheral nerve system revealed atrophy and demyelination of the *cauda equina*, and thinned sciatic and ulnar nerves heading towards it. The dorsal root ganglia were shrunken and atrophic, and stainings of the lumbar spinal cord and several afferent peripheral nerves confirmed a severe loss of myelinated fibers. In the severely affected areas close to the ankle less than one third of intact nerve fibers remained and densely packed columns of Schwann cells were left where the axons had retracted. Motoric nerve roots and fibers in the dorsal root ganglia however were preserved (15). Denny-Brown was convinced that the degenerated conditions of the dorsal root ganglia were the major cause of "hereditary perforating ulcer of the foot" and changed the name to "Hereditary sensory radicular neuropathy" (15). Dyck and colleagues later refined this descriptive term to hereditary sensory and autonomic neuropathy (HSAN) and classified five different subtypes of it (HSAN1-5) (10). Shared characteristics of all HSANs are depressed reflexes, altered pain and temperature perception, and the absence of normal axon flare in response to intradermal injection of histamine phosphate (16). Axonal nerve fiber loss of all sizes was reported in sural nerve biopsies where smaller unmyelinated fibers were even more affected (10).

Mutations in at least five genes are known to cause HSAN1. Each of these five genes is associated with a slightly distinct phenotype (5). The majority of HSAN1-associated mutations were identified in the sequences of two subunits of the enzyme serine palmitoyltransferase (SPT) (HSAN1A OMIM#162400 and HSAN1C OMIM#613640).

Furthermore, mutations in the sequences of the dynamin-related GTPase atlastin-1 (HSAN1D OMIM #613708), the DNA methyltransferase DNMT1 (HSAN1E OMIM #614116), and the RAS-related GTPase RAB7 (CMT 2B OMIM #600882 allelic with HSAN1B) are known to cause HSAN1 (5, 17).

HSAN1 is an autosomal dominant inherited neuropathy which facilitates the initial diagnosis based on family history. Almost every HSAN1 patient has at least one affected parent and will pass the disease down to 50% of his offspring (18). Penetrance of the phenotype is variable within the families, but also between different kinships with identical genotypes. Females were reported to present with reduced disease severity (11). An earlier hypothesis claimed better foot care and a reduced burden of heavy physical labor in women to be the reason for the lower tendency to develop foot ulcers (10).

The autosomal dominant inheritance is the most obvious factor to discriminate HSAN1 from HSAN2-5. The delayed onset between the 2nd and 5th decades, the slowly progressing course of the disease, and the preferential involvement of lower limbs are characteristic clinical markers for this subtype of HSAN (9, 10). Positive sensory symptoms such as shooting and burning pain and paraesthesia are also common and unique symptoms of HSAN1 (19).

Sensory loss predominantly affects lower limbs and develops in a characteristic glove and stocking pattern (see Figure 1-1) (19). Impaired pain and temperature sensation together with poor wound healing pave the way for unnoticed injuries and the frequent formation of ulcerating wounds, which can lead to gangrene and osteomyelitis and finally might necessitate the amputation of individual phalanxes or even whole limbs.

Variable motor involvement is usually present in advanced cases. Progressive muscle wasting and distal weakness can result in a mandatory use of walking aids and wheelchair dependency at a higher age. Autonomous disturbances such as sweating of the hands and feet can be present, but visceral signs of autonomic disturbances are rather rare.

Treatment of HSAN1 is symptomatic and focuses on the prevention of wounds and appropriate wound care to prevent ulcerations and associated complications. Ankle foot orthoses and arthrodesis can be useful to improve foot drop and more severe joint malformations such as Charcot joints. Medication with carbamazepine, gabapentin or pregabalin and amitryptiline is used to relieve patients of neuropathic pains and especially episodes of shooting pains during the night. (18).

1.4.2 Short description of HSAN types 2-7

Four other subtypes of HSAN (HSAN2-5) are all characterized by a recessive mode of inheritance, which is usually associated with an early onset and complete penetrance of the disease. Individual penetrance and severity of the reported symptoms are nevertheless highly variable (16). Only recently two more peripheral and autonomic neuropathies were annotated in the OMIM database as new phenotypes within the HSAN family, raising the number of subtypes to a total of seven (20, 21).

1.4.2.1 HSAN2

The two known isoforms of HSAN2 (HSAN2A OMIM #201300, and HSAN2B OMIM#613115) are caused by several mutations in WNK1 and FAM134B.Standardized genetic test kits for HSAN2 are not available.

HSAN2A (also known as Morvan´s disease) is a non-progressive congenital sensory neuropathy. It is characterized by an early childhood or even congenital onset, pronounced hypotonia, and universal sensory loss due to small fiber modalities. Autonomous dysfunctions occur early on and include neonatal feeding problems caused by poor oesophageal muscular coordination. The feeding problems cause recurring events of chocking and apnea. Early loss of distal pain-, temperature- and joint position-sensation causes complications such as unnoticed injuries and a self-mutilating behavior.

Thinning of the sural nerve and a decreased number of unmyelinated axons, together with absence of cutaneous receptors and nerve fibers, are pathological signs of HSAN2. (16).

1.4.2.2 HSAN3

HSAN type 3 (OMIM #223900), familial dysautonomia (FD) and Riley-Day syndrome are synonyms for the most common and best studied subtype of HSAN.

HSAN3 is caused by three different mutations in the gene IKBKAP, but only one of the three accounts for 99.5% of all known cases (22).

This is an autosomal recessive inherited disease which manifests exclusively in the progeny of the eastern European Jewish population (Ashkenazi Jews) (23). The carrier prevalence amongst individuals with Ashkenazi Jewish ancestry is 1 out of 27 (22). Kits for diagnostic testing of the IKBKAP variations are available and routinely used for family counselling and carrier testing.

HSAN3 presents with a congenital onset and progressive involvement of both the sensory and autonomic nervous system. Although there is no obvious dysmorphism at birth, affected individuals develop a characteristic facial expression over time. Characteristic flattening of

the upper lip, kyphoscoliosis, and a general short stature are the most obvious physical symptoms of HSAN3. Further cardinal symptoms for early diagnosis include alacrima (absence of tears during emotional crying) and feeding problems due to oral incoordination. Gastroesophagial reflux in combination with a reduced sensitivity to hypoxia cause and aggravate the frequent pneumological problems. The protracted occurrence of nausea and vomiting is also typical of HSAN3. These events of so-called dysautonomic crisis manifest with a cyclic (daily, weekly or monthly recurrence) pattern in 40% of the patients.

Sensory abnormalities are less pronounced in HSAN3 and self-mutilating behavior is rarely reported. Pain and temperature sensations are decreased but still present and although pain at skin and bones is poorly perceived, deep and visceral pain sensation is normal (16).

1.4.2.3 HSAN4

HSAN type 4 (OMIM #256800) is also known as congenital insensitivity to pain with anhydrosis (CIPA). HSAN4 is an autosomal recessive inherited disease. Several hundred cases of HSAN4 are known, making it the 2nd most frequent type of HSAN (16). HSAN4 is distributed equally amongst all ethnicities, but about 50% of the cases are associated with consanguinity. At least 37 different mutations of the neurotrophic tyrosine kinase receptor type 1 (NTRK1) are associated with HSAN4 (24), causing a more or less pronounced loss of function of the important NGF/TRKA neurotrophic signaling pathway. Due to the large number of associated mutations, commercial genetic testing is not available for HSAN4.

HSAN4 presents with congenital onset of first symptoms. One of the cardinal diagnostic features of HSAN4 is the marked decrease or even total absence of autonomous sweating. Frequent episodes of fevers and even hyperpyrexia in childhood occur as a consequence of the overall impaired thermal regulation, and represent characteristic early symptoms of HSAN4. Warm weather conditions therefore require the active cooling of the patients with cold water (25). Insensitivity to superficial but also deep visceral pains is another characteristic symptom of HSAN4 and gives rise to an increased prevalence of self-mutilations, unnoticed superficial injuries, and even multiple bone fractures (23, 25). Delayed development and low muscular strength and tone are frequently observed, but normalize during childhood. However, learning problems, hyperactivity, and emotional lability can be persistent complications of HSAN4 (16).

Nerve biopsies show an almost total absence of small unmyelinated fibers in peripheral nerves. Absence of appropriate innervation by these fibers adjacent to hypoplastic dermal sweat glands also explains the marked anhydrosis. However, small myelinated fibers are preserved in the sural nerve and a normal dermal network is still formed (10, 16).

1.4.2.4 HSAN5

HSAN type 5 (OMIM #608654) is an extremely rare autosomal recessive type of HSAN with only a few published case reports (23). HSAN5 is caused by mutations in the nerve growth factor beta (NGFB) (26).

In congruence with HSAN4, pain sensation is virtually absent in individuals with HSAN5, but temperature perception is only moderately impaired. Further sensory modalities such as perception of touch, pressure and vibration are usually not affected (10, 27). The onset of symptoms is congenital, and progressive aggravation or spreading of the symptoms has not been reported. Muscle strength, coordination, early childhood development and intelligence are normal.

Nerve biopsies show severe loss of thin myelinated fibers in the sural nerve, with a modest reduction of small unmyelinated fibers.

1.4.2.5 HSAN4 and HSAN5 are Allelic Disorders

A mild mutation in the sequence of NTRK1 was shown to cause the type 5 phenotype instead of the more severe type 4 (28). Recently a null mutation in NGFB was reported which extended the HSAN5 phenotype to encompass the HSAN4 characteristics as well (29). Therefore HSAN4 and 5 are very likely allelic disorders. Both are caused by mutations affecting the NGF/TRKA signaling pathway and their phenotypes are part of a phenotypic spectrum caused by variable impairment of this important neurotrophic pathway (28, 29). However, clear discrimination between the two subtypes is possible by determination of the characteristic pattern of the involved fibers, rather than by identification of the mutation only. HSAN4 is characterized by virtual absence of small unmyelinated fibers and preservation of myelinated fibers in the peripheral nerves. On the other hand, the HSAN5 phenotype is associated with severe reductions of myelinated fibers and only moderate involvement of the unmyelinated ones. The presence of small unmyelinated fibers in the peripheral nerves therefore seems to make the difference (28).

1.4.2.6 HSAN6

HSAN6 (OMIM #614653) is caused by mutations in the large cytoskeletal linker protein dystonin (DST). HSAN6 is a recessively inherited peripheral autonomic neuropathy with lethal course.

The three recently reported affected infants of a large consanguineous kinship of Ashkenazi Jewish descent showed all characteristics of HSAN3, but in addition presented with a motionless and opened-mouthed facial expression, severe psychomotor retardation, distal contractures, and early death (after 9, 22 and 24 months). Further analysis of the DST

mutation in mice revealed the instability of the mutated transcript, resulting in cytoskeletal disorganization. Fibroblasts of HSAN3 patients with the IKBKAP mutation however presented 6-fold increased protein concentration of neural dystonin. This suggests an important attenuating effect of DST on the milder HSAN3 symptoms. Furthermore this explains and highlights the close phenotypical relationship of HSAN3 and the newly described dystonin-associated HSAN6 (21).

1.4.2.7 HSAN7

HSAN7 (OMIM # 615548) is caused by a mutation in SCN11A and does not fit into the classical HSAN categories (10, 30). Both reported individuals carry the heterozygous *de novo* mutation in the voltage gated sodium channel SCN11A, which is expressed mainly in nociceptors.

Symptoms presented by the patients include congenital inability to experience pain resulting in severe self-mutilation and multiple unnoticed fractures. Slow wound healing, mild muscular weakness and delayed motor development were observed as well. Both patients presented with a prominent hyperhidrosis which allows immediate differentiation of their phenotype from HSAN3, HSAN4 and HSAN5 which all cause anhydrosis with variable extent.

Motor and sensory nerve conduction velocities were slightly decreased in these patients, but neither muscle biopsy, nor EMG showed abnormalities. Nerve biopsies revealed no sensory axonal loss, and patients also did not have intellectual disabilities (20).

1.5 Mutations in SPT cause HSAN1

Until today mutations in five different genes have been identified and conclusively associated with HSAN1 (5). The majority of HSAN1-causing mutations are located in the sequences of two subunits of the enzyme serine palmitoyltransferase (SPT) (see Figure 1-3).

Soon after mapping the first causative gene for HSAN1 to chromosome 9q22.1-q22.3 (31), this mapping was refined to the first three point mutations (c.399T→G ➔ p.C133W; c.398G→A ➔ p.C133Y and c.432T→A ➔ p.V144D) found in the sequence of the serine palmitoyltransferase long chain base subunit-1 (SPTLC1) (32, 33).

Verhoeven reported another mutation in SPTLC1 (c.1160G→C ➔ p.G387A) to be associated with two quite severe cases of mutilating HSAN1 in twin sisters (34). We could show by biochemical and also segregation analysis that p.G387A cannot be the sole cause for the observed pathology in these patients, and the initial annotation as an HSAN1-causing mutation was incorrect (35).

In 2009 Rotthier et al. performed a larger genetic screening in a cohort of 100 patients assigned to any subtype of a hereditary sensory and autonomic neuropathy. For the autosomal dominant inherited subtype of HSAN1, they focused on the two genes for SPTLC1 and Rab7 and were able to identify two unknown mutations in SPTLC1 in two isolated cases (p.A352V and p.S331F). The exchange of serine for phenylalanine at position 331 of SPTLC1 occurred *de novo* in the index case which presented with a severe phenotype with congenital onset, and growth and mental retardation (36).

In 2010 Rotthier et al. reported three HSAN1-causing mutations in SPTLC2 (c.1145G→T ➔ p.G382V; c.1075G→A ➔ p. V359M and c.1510A→T ➔ p.I504F) (37).

Only recently Michaela Auer-Grumbach identified a new mutation at p.331 in SPTLC1 (p.S331Y) (38). The mutation occurred *de novo* and the index case presented with a severe phenotype with early onset, pronounced muscular hypertrophy, reduced peripheral pain sensation and hypermobility and deformation of joints, resembling the symptoms described in the two previously reported p.S331F patients (36, 39, 40).

In 2013, Murphy and colleagues reported a mutation in SPTLC2 causing an exchange of alanine for proline at position 182 (p.A182P) (41). The two examined index cases carrying the mutation presented with an early onset (5 and 10y) and severe muscle wasting in upper and lower limbs. The same group recently identified another mutation in SPTLC2 at position 384 (p.S384F) which was conclusively associated with the HSAN1 phenotype (42, 43).

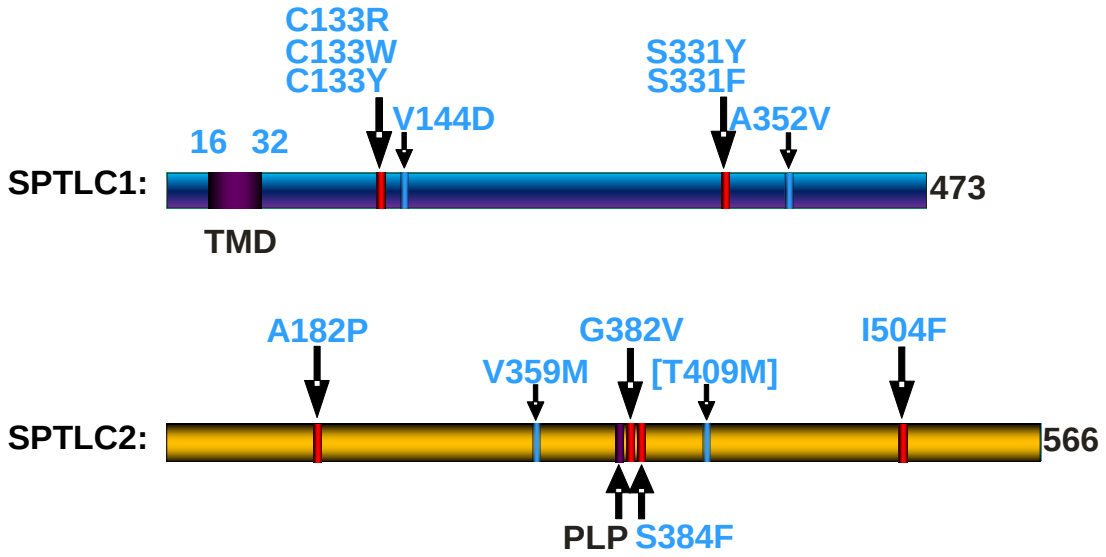


Figure 1-3 HSAN1-associated mutations in SPTLC1 and SPTLC2
TMD = functional trans-membrane domain, PLP = pyridoxal 5 phosphate binding motif, red bars indicate mutations which increase 1-deoxySL formation *in vitro,* parentheses indicate not yet published mutations

1.6 SPT and *de novo* synthesis of SL and 1-deoxySL

Serine palmotoyltransferase (SPT) catalyzes the condensation of L-serine with the activated fatty acid palmitoyl-CoA. This initial and rate limiting step of sphingolipid *de novo* synthesis takes place at the outer membrane of the endoplasmatic reticulum (44, 45).

SPT is a member of the family of pyridoxal 5'-phosphate (PLP) dependent α-oxoamine synthases (POAS) (46). Besides SPT, the POAS family comprises three more enzymes: 5-amino-levulinic acid synthase, 2-amino-3-ketobutyrate ligase (KBL), and 8-amino-7-oxononanoate synthase (AONS) which all are expressed as soluble homodimers (47).

A lot of work on SPT has been conducted in the model organism *Saccharomyces cerevisiae*. In yeast, the functional SPT complex consists of at least two subunits called long chain base 1 and 2 (LCB1 and LCB2, respectively). The amino acid sequences of LCB1 and LCB2 present 21.6% identity and 43% similarity (48, 49). Both LCB subunits carry the PLP binding motif [G^{362}TFT**K**SFG^{369}], which was identified in the other PAOS members as well (50, 51). But unlike in LCB2 and the other POAS, in the LCB1 sequence the highly conserved lysine 366 residue is exchanged for threonine, which makes the formation of a Schiff base with the PLP cofactor impossible. Therefore LCB1 was suggested to play a regulatory role, while LCB2 binds the cofactor and lends catalytic activity to the complex (49). The catalytic active complex however is formed upon dimerization of both subunits, which forms the active center (52).

In mammalian cells, three subunits of SPT have been identified (45, 53). The human serine palmitoyltransferase long chain base subunit 1 (SPTLC1) is a 53kDa protein with a putative N-terminal trans-membrane domain. The second subunit SPTLC2 is larger (63kDa) and its amino acid sequence shows 29% identity and 52% similarity to SPTLC1 (45).
The third subunit of the mammalian SPT (SPTLC3) equals SPTLC2 in size (63kDa) and shows 68% identity and 84% similarity to SPTLC2, but only 21% and 45% respectively to SPTLC1 (53). All three major subunits of mammalian SPT carry a highly hydrophobic N-terminal trans membrane domain, but only the highly identical subunits SPTLC2 and SPTLC3 present the conserved motif (T[FL][GTS]**K**[SAG][FLV]G) for covalent binding of the PLP cofactor and catalytic activity (47).

The three subunits of mammalian SPT are suspected to form a larger hetero-octameric complex with a mass of 460-480kDa (54). Each of the four heterodimers within that complex consists of one SPTLC1 subunit and either an SPTLC2 or SPTLC3 subunit, with the PLP-cofactor localizing within the active center of the dimer.Therefore it has been suggested that SPTLC1 might function as an integral membrane anchor, which binds SPTLC2 and 3 and stabilizes the cytosolic orientation of their catalytic active sites (54).

The initial reaction catalyzed by SPT is the condensation of an activated fatty acid (usually palmitoyl CoA) and the amino acid L-serine (for an overview see Figure 1-4). This condensation reaction forms the intermediate sphingoid base 3-ketosphinganine, which is rapidly reduced by the NADPH-dependent 3-ketosphinganine reductase. This step results in the formation of sphinganine (or dihydrosphingosine), the first stable sphingoid base. Ceramide synthases catalyze the attachment of the second fatty acid via an amide bond, and thereby give rise to the formation of dihydroceramide. Ceramide synthases are expressed in six isoforms which have specific preferences for certain fatty acid-CoAs. The enzyme dihydroceramide desaturase converts the dihydroceramide to ceramide by introduction of a 4-5 trans double bond into the sphinganine backbone. Ceramide, and to a lesser extent the intermediate compound dihydroceramide, are necessary precursors for the formation of the more complex glycosphingolipids and sphingomyelins. Ceramides can be degraded by the action of ceramidase, which cleaves off the N-linked fatty acid. This results in the free sphingoid base sphingosine, which is ultimately degraded by the combined action of sphingosine-1-phosphate kinase and S1P-lyase.

Palmitoyl-CoA is the preferred substrate for SPT and also the most abundant acyl-CoA in mammalian cells. However, the enzyme can use other acyl-CoAs for the initiation of SL *de novo* synthesis *in vitro* as well (55–58). Use of the rare odd-numbered pentadecanoyl- and heptadecanoyl-CoAs in the condensation reaction thereby results in formation of C17 and C19 sphingoid bases, while the even-numbered myristoyl and stearoyl-CoA are processed to become C16 and C20-based sphingolipids, respectively.

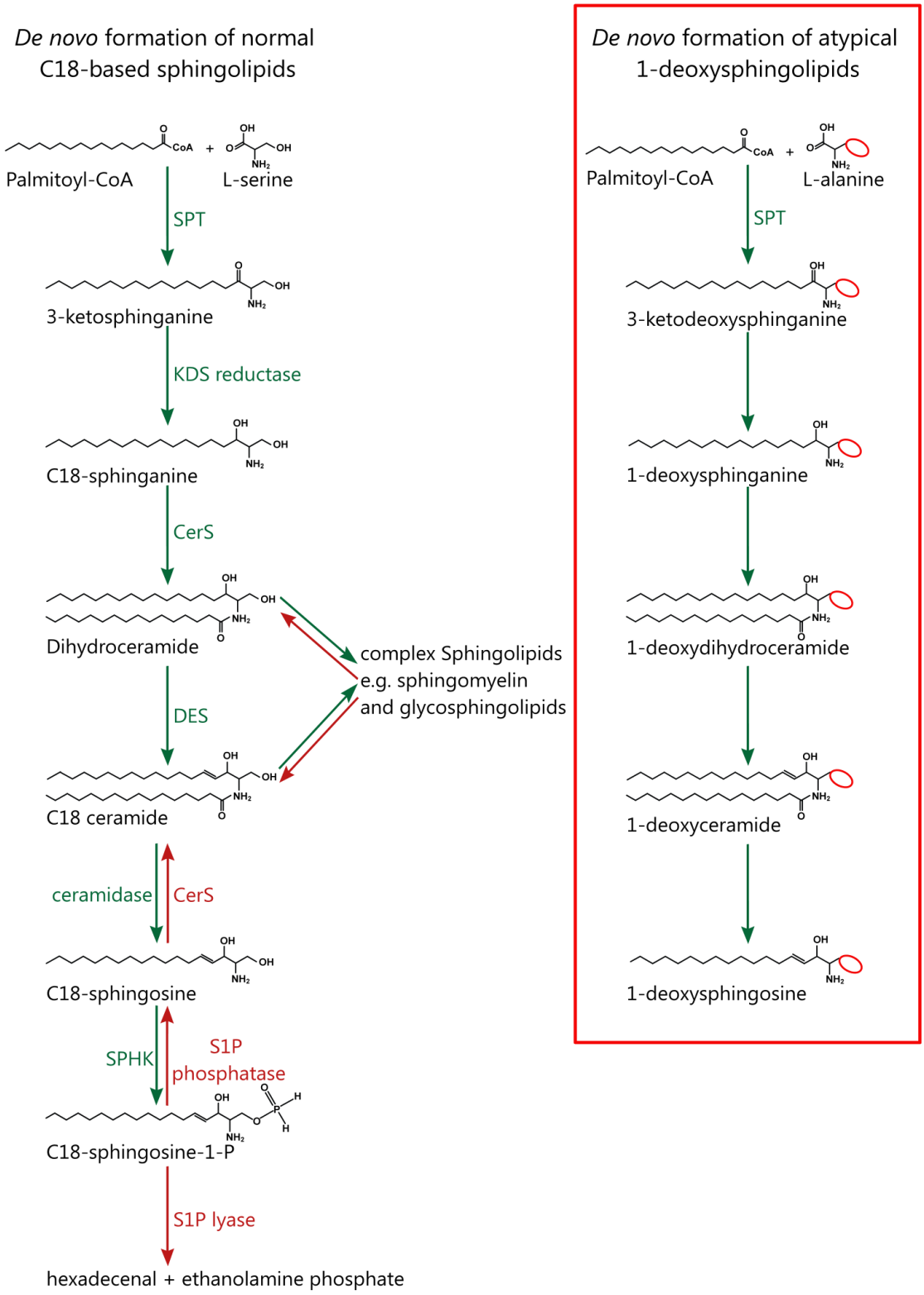


Figure 1-4 Pathways for *de novo* synthesis of C18-based sphingolipids and the atypical 1-deoxysphingolipids 1-deoxysphinganine and 1-deoxysphingosine. Synthesis processes are indicated by green arrows with the responsible enzymes annotated next to them. Several steps are reversible, as indicated by red arrows. Final cleavage of C18-sphingosine-1-phosphate is irreversible and not possible for the atypical 1-deoxySL. SPT = serine palmitoyltransferase; KDS = 3-ketodihydrosphinganine; CerS = ceramides synthase; DES = dihydroceramide desaturase; SPHK = sphingosine kinase; S1P = sphingosine 1-phosphate

After the identification of mutations in SPT as a cause for HSAN1, Dawkins and colleagues reported an increased *de novo* synthesis of glycosylated ceramides which was believed to be caused by the mutated SPT and to result in increased apoptosis of the cells (33).
This educated guess was refuted by more recent studies, describing reduced overall SPT activity and active inhibition of normal SPT function by overexpression of the mutated subunits (59).

Gable and colleagues obtained similar results when they expressed the corresponding mutations in the Lcb1 subunit of yeast SPT. Modelling the structure of the yeast SPT complex (heterodimer from Lcb1 and 2) suggested a localization of the three mutations at the interface of the two subunits. The mutation sites were in close proximity to the active PLP-binding domain on the Lcb2 subunit and were suggested to play a crucial role in conferring the dominant negative effects on the activity of the complex (60).

Although initially denied (56), it has been shown that SPT can also metabolize other amino acids besides its canonical substrate L-serine. Zitomer and colleagues identified a new sphingolipid metabolite which arises from the SPT catalyzed condensation of L-alanine and palmitoyl-CoA (61). The atypical 1-deoxysphinganine (1-deoxySA) accumulated almost exclusively in cells treated with the CerS inhibitor Fumonisin B1 (FB1). Toxicity tests revealed an increased cytotoxicity of 1-deoxySA on a variety of cancer cell lines and suggested an important role in FB1-related pathologies (61).

Anke Penno reported a shift of the substrate preference of SPT caused by the HSAN1 mutations (62). The mutations in SPTLC1 increased the activity of the SPT complex with two alternative amino acid substrates, L-alanine and glycine, giving rise to the increased formation of the atypical sphingoid bases (SB) 1-deoxysphinganine and 1-desoxymethylsphinganine, respectively (see Figure 1-4). In the absence of FB1, these 1-deoxysphingoid bases are further acylated to N-acyl-1-deoxydihydroceramides and finally become 1-deoxyceramides. This new class of atypical sphingolipids is named 1-deoxysphingolipids (1-deoxySL or dSL). Due to the missing C_1 hydroxyl group, 1-deoxysphingolipids can neither be processed to the more complex sphingomyelins and glycosphingolipids via attachment of headgroups like phosphocholine and sugar moieties, nor degraded, as the canonical degradation pathway for sphingoid bases requires phosphorylation of C_1 to form the catabolic intermediate sphingosine-1-phosphate (62).

Elevated 1-deoxysphingolipids were found in HEK293 cells expressing the mutated SPT subunits, but also in lymphoblasts and plasma from HSAN1 patients as compared to wild type (wt) cells or plasma of healthy controls. Neurotoxic effects of 1-deoxysphinganine were described in two phase 1 chemotherapeutic studies but also observed in cultured primary

neurons (62–64). Therefore, Penno et al. concluded that HSAN1 is actually caused by a gain of function of SPT, resulting in the slow accumulation of neurotoxic sphingolipids within the peripheral nerves and causing their slow decay (62).

Increasing numbers of HSAN1-associated mutations in SPTLC1 but also SPTLC2 have been identified over the last years in neuropathy patients and conclusively associated with the disease. Elevated levels of 1-deoxySL were consistently found in the plasma of all HSAN1 patients bearing a mutation in SPT, and therefore represent an important hallmark of this kind of neuropathy.

In 2010 Rotthier et al. reported elevated concentrations of 1-deoxySLs in cells expressing one of the three new HSAN1 mutations in SPTLC2 and in the plasma of the patients, which confirmed the accumulation of 1-deoxysphinganine as the most conclusive cause of HSAN1 (37).

In 2011 two of the mutations in SPTLC1 (p.A352V and p.S331F) which cause a distinct HSAN1 phenotype were characterized in more detail. Rotthier et al. reported reduced canonical SPT activity *in vitro* and the accumulation of 1-deoxySLs in plasma as common features of both mutations, but only the overexpression of p.S331F increased activity with alanine in HEK293 cells (Rotthier et al., 2011).

Besides the pronounced phenotypical similarities between p.S331F and p.S331Y, the biochemical properties of these two mutations in SPTLC1 presented a high degree of congruence. Both mutations showed an increased activity with L-alanine but also with the canonical substrate L-serine. The two mutations at Ser 331 strongly imply a direct link between the position of the mutated residue and the biochemical properties of the resulting SPT complexes. The very distinct phenotype associated with these mutations argues for additional pathological changes associated with mutations at this position (38).

In 2013 Murphy et al. reported highly elevated 1-deoxySL levels in the plasma of the patients bearing the new SPTLC2 p.A182P mutation. Furthermore, the biochemical analysis of the mutated SPTLC2 subunit revealed an exceptionally high increase in its activity with alanine (15-fold increase), while its activity with serine was considerably (2-3-folds) decreased. The study confirmed the strong causative association of 1-deoxySL production in vitro and elevated 1-deoxySL levels in HSAN1 (41).

1.7 Serine Therapy

While diagnostics and classification of rare inherited disorders such as HSAN have improved a lot over the last decade, therapeutic treatment strategies for most sensory neuropathies are still mostly supportive and seek to ameliorate the severity or prevent progression of the symptoms rather than interfering with the underlying pathological mechanisms.

After the identification of mutations in SPTLC1 and SPTLC2 as causes for HSAN1A and 1C, more effort was put into the elucidation of the relationship between sphingolipid metabolism and HSAN1. The discovery of elevated concentrations of the neurotoxic atypical 1-deoxySLs in plasma and cells of HSAN1A and 1C patients (62) closed this gap. Understanding SPT´s canonical substrates and products, as well as the alternatively formed atypical and neurotoxic products, led to the idea of inhibitting 1-deoxySL formation by increasing the availability of L-serine. First studies in human cell lines overexpressing mutant SPTLC1 confirmed the 1-deoxySL-lowering effect of high doses of L-serine supplementation. Feeding of an L-serine enriched diet was shown to effectively protect transgenic mice (SPTLC1 p.C133W) from onset of neuropathy symptoms. In contrast, enrichment of the diet with L-alanine for two months resulted in an earlier onset of neuropathy and a marked aggravation of the symptoms (65). A pilot study on the effects of oral L-serine supplementation in fourteen HSAN1 patients bearing the p.C133Y variant of SPTLC1 confirmed the findings from the mouse model (p.C133W). The 1-deoxySL concentrations in plasma of the patients were reduced within two weeks, stabilized at a low level, and started to rise again during the washout phase of the trial. Although neurological improvements were not assessed in this short study, the participants reported improvements of their health status such as increased sensibility and tingling feeling in the hands, improvements in skin robustness, and nail and hair growth (65). The potential of L-serine supplementation as a therapy in HSAN1 is now being followed up in a 2 year double blinded clinical study (clinical trial no. NCT01733407).

1.8 Genotypic and phenotypic variability of HSAN1

Patients with mutations in SPT frequently present with spontaneous lancinating or shooting pains in the lower limbs, which differentiates them clearly from patients with other HSAN1 genotypes (9, 17).

The five identified causative genes for HSAN1 are responsible for distinct functions and not related to each other. Mutations within the sequences of all of these genes, and even several mutations within the same gene (e.g. seven in SPTLC1 and six in SPTLC2), cause the HSAN1 phenotype.

This collective phenotype however is highly variable. The average age of onset and the degree of autonomic and motor involvement vary markedly for different mutations of the same gene and even between different patients bearing the same mutation.

The putative neurotoxic 1-deoxySLs are not only formed by mutated SPT, but also by the wild type enzyme, and low 1-deoxySL levels can be detected in the plasma of healthy controls, although at minimal concentrations. Increased concentrations of 1-deoxySLs can be used as a biomarker for HSAN1A and HSAN1C. Recently we reported significantly elevated levels of 1-deoxySLs in patients with metabolic syndrome or type 2 diabetes mellitus (T2DM) (66, 67). Diabetes mellitus causes a peripheral neuropathy which clinically highly resembles HSAN1. Understanding the mechanisms leading to the formation of 1-deoxySLs and the underlying pathomechanism therefore might also improve the understanding of lifestyle diseases such as T2DM and other metabolic disorders and their sequelae.

1.9 Objectives of the Thesis

The objective of this work was to improve the understanding of the biochemical basis of HSAN1A and HSAN1C. In this work we provide a comprehensive analysis of all HSAN1-associated mutations. We compared the biochemical characteristics of the mutants in standardized assays which allow the fast addition of new mutations to this comparison chart. We observed a clear genotype-phenotype correlation for several HSAN1 mutations, which allowed us to group the mutants and also to predict the phenotype from the genomic information to a certain extent.

In addition we analyzed an experimental serine therapy in the case of an S331F patient with an exceptionally severe phenotype. We observed a multitude of subjective and clinical improvements even in this severely affected patient, which again emphasizes the potential of L-serine to not only attenuate the progression of the symptoms but to ameliorate the symptoms in HSAN1.

1.10 References for general introduction:

1. Willison, H. J. (2003) CLINICAL EVALUATION AND INVESTIGATION OF NEUROPATHY. *J. Neurol. Neurosurg. Psychiatry*. 74, 3ii–8

2. Thomas, P. K., and Ochoa, J. (1993) Clinical features and differential diagnosis. in *Peripheral Neuropathy*, 3rd Editio (Dyck, P. J., Thomas, K. P., Griffin, J. W., Low, P. A., and Poduslo, J. F. eds), pp. 749–774, Saunders Philladelphia

3. Thomas, P. K. (1970) Peripheral neuropathy. *Br. Med. J.* 1, 349–351

4. Dyck, P. J., Oviatt, K. F., and Lambert, E. H. (1981) Intensive evaluation of referred unclassified neuropathies yields improved diagnosis. *Ann. Neurol.* 10, 222–6

5. Rotthier, A., Baets, J., Timmerman, V., and Janssens, K. (2012) Mechanisms of disease in hereditary sensory and autonomic neuropathies. *Nat. Rev. Neurol.* 8, 73–85

6. Reilly, M. M. (2007) Sorting out the inherited neuropathies. *Pract. Neurol.* 7, 93–105

7. Rossor, A. M., Polke, J. M., Houlden, H., and Reilly, M. M. (2013) Clinical implications of genetic advances in Charcot-Marie-Tooth disease. *Nat. Rev. Neurol.* 9, 562–71

8. Reilly, M. M. (2002) Genetic neuromuscular disease. *J. Neurol. Neurosurg. Psychiatry*. 73, 12ii–21

9. Auer-Grumbach, M. (2008) Hereditary sensory neuropathy type I. *Orphanet J. Rare Dis.* 3, 7

10. Dyck, P. J. (1993) Neuronal atrophy and degeneration predominantly affecting peripheral sensory and autonomic neurons. in *Peripheral Neuropathy*, 3rd Editio (Dyck, P. J., Thomas, K. P., Griffin, J. W., Low, P. A., and Poduslo, J. F. eds), pp. 1065–1093, Saunders Philladelphia

11. Houlden, H., King, R., Blake, J., Groves, M., Love, S., Woodward, C., Hammans, S., Nicoll, J., Lennox, G., O'Donovan, D. G., Gabriel, C., Thomas, P. K., and Reilly, M. M. (2006) Clinical, pathological and genetic characterization of hereditary sensory and autonomic neuropathy type 1 (HSAN I). *Brain*. 129, 411–25

12. Leplat, M. (1846) Dictionnaire de medecine en 30 volumes. *Paris*. [online] http://scholar.google.de/scholar?hl=de&q=Leplat+M.+Dictionnaire+de+Medecine+en+30+volumes.+Paris+1846&btnG=Senden&lr=#0#0 (Accessed April 22, 2014)

13. Nelaton, M. (1852) Affection singuliere des os du pied. *Gaz Hop Civ. Mil.* [online] http://scholar.google.de/scholar?hl=de&q=++Nelaton+M+(1852)+Affection+singuliere+des+os+du+pied.+Gaz++Hop+Civils+Militaires+4,+13–22.&btnG=Senden&lr=#0#0 (Accessed April 22, 2014)

14. Hicks, E. P. (1922) HEREDITARY PERFORATING ULCER OF THE FOOT. *Lancet*. 199, 319–321

15. Denny-Brown, D. (1951) Hereditary sensory radicular neuropathy. *J. Neurol. Neurosurg. Psychiatry*. [online] http://jnnp.bmj.com/content/14/4/237 (Accessed September 3, 2012)

16. Axelrod, F. B., and Gold-von Simson, G. (2007) Hereditary sensory and autonomic neuropathies: types II, III, and IV. *Orphanet J. Rare Dis.* 2, 39

17. Klein, C. J., Duan, X., and Shy, M. E. (2013) Inherited neuropathies: Clinical overview and update. *Muscle Nerve*. 10.1002/mus.23775

18. Nicholson, G. A. (2014) HSANIA. *NCBI Gene Rev.*

19. Houlden, H., King, R., Blake, J., and Groves, M. (2006) Clinical, pathological and genetic characterization of hereditary sensory and autonomic neuropathy type 1 (HSAN I). *Brain*. 1, 411–425

20. Leipold, E., Liebmann, L., Korenke, G. C., Heinrich, T., Giesselmann, S., Baets, J., Ebbinghaus, M., Goral, R. O., Stödberg, T., Hennings, J. C., Bergmann, M., Altmüller, J., Thiele, H., Wetzel, A., Nürnberg, P., Timmerman, V., De Jonghe, P., Blum, R., Schaible, H.-G., Weis, J., Heinemann, S. H., Hübner, C. a, and Kurth, I. (2013) A *de novo* gain-of-function mutation in SCN11A causes loss of pain perception. *Nat. Genet.* 45, 1399–404

21. Edvardson, S., Cinnamon, Y., Jalas, C., Shaag, A., Maayan, C., Axelrod, F. B., and Elpeleg, O. (2012) Hereditary sensory autonomic neuropathy caused by a mutation in dystonin. *Ann. Neurol.* 71, 569–72

22. Axelrod, F. B. (2006) A world without pain or tears. *Clin. Auton. Res.* 16, 90–7

23. Hilz, M. J. (2002) Assessment and evaluation of hereditary sensory and autonomic neuropathies with autonomic and neurophysiological examinations. *Clin. Auton. Res.* 12 Suppl 1, I33–43

24. Indo, Y. (2001) Molecular basis of congenital insensitivity to pain with anhidrosis (CIPA): mutations and polymorphisms in TRKA (NTRK1) gene encoding the receptor tyrosine kinase for nerve growth factor. *Hum. Mutat.* 18, 462–71

25. Swanson, A. G. (1963) Congenital Insensitivity to Pain with Anhydrosis. *Arch. Neurol.* 8, 299

26. Einarsdottir, E., Carlsson, A., Minde, J., Toolanen, G., Svensson, O., Solders, G., Holmgren, G., Holmberg, D., and Holmberg, M. (2004) A mutation in the nerve growth factor beta gene (NGFB) causes loss of pain perception. *Hum. Mol. Genet.* 13, 799–805

27. Low, P. a, Burke, W. J., and McLeod, J. G. (1978) Congenital sensory neuropathy with selective loss of small myelinated fibers. *Ann. Neurol.* 3, 179–82

28. Houlden, H., King, R. H., Hashemi-Nejad, A., Wood, N. W., Mathias, C. J., Reilly, M., and Thomas, P. K. (2001) A novel TRK A (NTRK1) mutation associated with hereditary sensory and autonomic neuropathy type V. *Ann. Neurol.* 49, 521–5

29. Carvalho, O. P., Thornton, G. K., Hertecant, J., Houlden, H., Nicholas, A. K., Cox, J. J., Rielly, M., Al-Gazali, L., and Woods, C. G. (2011) A novel NGF mutation clarifies the molecular mechanism and extends the phenotypic spectrum of the HSAN5 neuropathy. *J. Med. Genet.* 48, 131–5

30. Klein, C. J., Wu, Y., Kruckeberg, K. E., Hebbring, S. J., Anderson, S. a, Cunningham, J. M., Dyck, P. J. B., Klein, D. M., Thibodeau, S. N., and Dyck, P. J. (2005) SPTLC1 and RAB7 mutation analysis in dominantly inherited and idiopathic sensory neuropathies. *J. Neurol. Neurosurg. Psychiatry*. 76, 1022–4

31. Nicholson, G. A., Dawkins, J. L., Blair, I. P., Kennerson, M. L., Gordon, M. J., Cherryson, A. K., Nash, J., and Bananis, T. (1996) The gene for hereditary sensory neuropathy type I (HSN-I) maps to chromosome 9q22.1-q22.3. *Nat. Genet.* 13, 101–4

32. Bejaoui, K., Wu, C., Scheffler, M. D., Haan, G., Ashby, P., Wu, L., de Jong, P., and Brown, R. H. (2001) SPTLC1 is mutated in hereditary sensory neuropathy, type 1. *Nat. Genet.* 27, 261–2

33. Dawkins, J. L., Hulme, D. J., Brahmbhatt, S. B., Auer-Grumbach, M., and Nicholson, G. a (2001) Mutations in SPTLC1, encoding serine palmitoyltransferase, long chain base subunit-1, cause hereditary sensory neuropathy type I. *Nat. Genet.* 27, 309–12

34. Verhoeven, K., Coen, K., De Vriendt, E., Jacobs, A., Van Gerwen, V., Smouts, I., Pou-Serradell, A., Martin, J.-J., Timmerman, V., and De Jonghe, P. (2004) SPTLC1 mutation in twin sisters with hereditary sensory neuropathy type I. *Neurology*. 62, 1001–1002

35. Hornemann, T., Penno, A., Richard, S., Nicholson, G., van Dijk, F. S., Rotthier, A., Timmerman, V., and von Eckardstein, A. (2009) A systematic comparison of all mutations in hereditary sensory neuropathy type I (HSAN I) reveals that the G387A mutation is not disease associated. *Neurogenetics*. 10, 135–43

36. Rotthier, A., Baets, J., De Vriendt, E., Jacobs, A., Auer-Grumbach, M., Lévy, N., Bonello-Palot, N., Kilic, S. S., Weis, J., Nascimento, A., Swinkels, M., Kruyt, M. C., Jordanova, A., De Jonghe, P., and Timmerman, V. (2009) Genes for hereditary sensory and autonomic neuropathies: a genotype-phenotype correlation. *Brain*. 132, 2699–711

37. Rotthier, A., Auer-Grumbach, M., Janssens, K., Baets, J., Penno, A., Almeida-Souza, L., Van Hoof, K., Jacobs, A., De Vriendt, E., Schlotter-Weigel, B., Löscher, W., Vondráček, P., Seeman, P., De Jonghe, P., Van Dijck, P., Jordanova, A., Hornemann, T., and Timmerman, V. (2010) Mutations in the SPTLC2 subunit of serine palmitoyltransferase cause hereditary sensory and autonomic neuropathy type I. *Am. J. Hum. Genet.* 87, 513–22

38. Auer-Grumbach, M., Bode, H., Pieber, T. R., Schabhüttl, M., Fischer, D., Seidl, R., Graf, E., Wieland, T., Schuh, R., Vacariu, G., Grill, F., Timmerman, V., Strom, T. M., and Hornemann, T. (2013) Mutations at Ser331 in the HSN type I gene SPTLC1 are associated with a distinct syndromic phenotype. *Eur. J. Med. Genet.* 56, 266–9

39. Huehne, K., Zweier, C., Raab, K., Odent, S., Bonnaure-Mallet, M., Sixou, J.-L., Landrieu, P., Goizet, C., Sarlangue, J., Baumann, M., Eggermann, T., Rauch, A., Ruppert, S., Stettner, G. M., and Rautenstrauss, B. (2008) Novel missense, insertion and deletion

mutations in the neurotrophic tyrosine kinase receptor type 1 gene (NTRK1) associated with congenital insensitivity to pain with anhidrosis. *Neuromuscul. Disord.* 18, 159–66

40. Rotthier, A., Penno, A., Rautenstrauss, B., Auer-Grumbach, M., Stettner, G. M., Asselbergh, B., Van Hoof, K., Sticht, H., Lévy, N., Timmerman, V., Hornemann, T., and Janssens, K. (2011) Characterization of two mutations in the SPTLC1 subunit of serine palmitoyltransferase associated with hereditary sensory and autonomic neuropathy type I. *Hum. Mutat.* 32, E2211–25

41. Murphy, S. M., Ernst, D., Wei, Y., Laurà, M., Liu, Y.-T., Polke, J., Blake, J., Winer, J., Houlden, H., Hornemann, T., and Reilly, M. M. (2013) Hereditary sensory and autonomic neuropathy type 1 (HSANI) caused by a novel mutation in SPTLC2. *Neurology*. 80, 2106–11

42. Ernst, D. (2013) Regulation of Deoxy-Sphingolipids and Their Role in Disease. *PhD Thesis*

43. Ernst, D., Murphy, S. M., Sathiyanadan, K., Wei, Y., Othman, A., Laura, M., Donaghy, M., Houlden, H., Reilly, M. M., and Hornemann, T. (2015) Novel HSAN1 Mutation in Serine Palmitoyltransferase Resides at a Putative Phosphorylation Site That Is Involved in Regulating Substrate Specificity. *NeuroMolecular Med.* 17, 47–57

44. Hanada, K., Hara, T., Nishijima, M., Kuge, O., Dickson, R. C., and Nagiec, M. M. (1997) A mammalian homolog of the yeast LCB1 encodes a component of serine palmitoyltransferase, the enzyme catalyzing the first step in sphingolipid synthesis. *J. Biol. Chem.* 272, 32108–14

45. Weiss, B., and Stoffel, W. (1997) Human and murine serine-palmitoyl-CoA transferase--cloning, expression and characterization of the key enzyme in sphingolipid synthesis. *Eur. J. Biochem.* 249, 239–47

46. Yard, B. a, Carter, L. G., Johnson, K. a, Overton, I. M., Dorward, M., Liu, H., McMahon, S. a, Oke, M., Puech, D., Barton, G. J., Naismith, J. H., and Campopiano, D. J. (2007) The structure of serine palmitoyltransferase; gateway to sphingolipid biosynthesis. *J. Mol. Biol.* 370, 870–86

47. Hanada, K. (2003) Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids*. 1632, 16–30

48. Buede, R., Rinker-Schaffer, C., Pinto, W. J., Lester, R. L., and Dickson, R. C. (1991) Cloning and characterization of LCB1, a Saccharomyces gene required for biosynthesis of the long-chain base component of sphingolipids. *J. Bacteriol.* 173, 4325–32

49. Nagiec, M. M., Baltisberger, J. a, Wells, G. B., Lester, R. L., and Dickson, R. C. (1994) The LCB2 gene of Saccharomyces and the related LCB1 gene encode subunits of serine palmitoyltransferase, the initial enzyme in sphingolipid synthesis. *Proc. Natl. Acad. Sci. U. S. A.* 91, 7899–902

50. Mukherjee, J. J., and Dekker, E. E. (1990) 2-Amino-3-ketobutyrate CoA ligase of Escherichia coli: stoichiometry of pyridoxal phosphate binding and location of the pyridoxyllysine peptide in the primary structure of the enzyme. *Biochim. Biophys. Acta*. 1037, 24–9

51. Alexeev, D., Alexeeva, M., Baxter, R. L., Campopiano, D. J., Webster, S. P., and Sawyer, L. (1998) The crystal structure of 8-amino-7-oxononanoate synthase: a bacterial PLP-dependent, acyl-CoA-condensing enzyme. *J. Mol. Biol.* 284, 401–19

52. Ikushiro, H., Islam, M. M., Tojo, H., and Hayashi, H. (2007) Molecular characterization of membrane-associated soluble serine palmitoyltransferases from Sphingobacterium multivorum and Bdellovibrio stolpii. *J. Bacteriol.* 189, 5749–61

53. Hornemann, T., Richard, S., Rütti, M. F., Wei, Y., von Eckardstein, A., Serine-palmitoyltransferase, M., Ru, M. F., and Eckardstein, A. Von (2006) Cloning and initial characterization of a new subunit for mammalian serine-palmitoyltransferase. *J. Biol. Chem.* 281, 37275–81

54. Hornemann, T., Wei, Y., and Eckardstein, A. V. O. N. (2007) Is the mammalian serine palmitoyltransferase a high-molecular-mass complex ? 164, 157–164

55. Williams, D., and Merrill, H. (1984) Enzymology of Long-Chain Base Synthesis by Liver : Characterization Serine Palmitoyltransferase in Rat Liver Microsomes. *Arch. Biochem. Biophys.* 228, 282–291

56. Hanada, K., Hara, T., and Nishijima, M. (2000) Purification of the serine palmitoyltransferase complex responsible for sphingoid base synthesis by using affinity peptide chromatography techniques. *J. Biol. Chem.* 275, 8409–15

57. Braun, P. E., Morell, P., and Radin, N. S. (1970) Synthesis of C18- and C20-dihydrosphingosines, ketodihydrosphingosines, and ceramides by microsomal preparations from mouse brain. *J. Biol. Chem.* 245, 335–41

58. Merrill, A. H. (1983) Characterization of serine palmitoyltransferase activity in Chinese hamster ovary cells. *Biochim. Biophys. Acta*. 754, 284–91

59. Bejaoui, K., Uchida, Y., Yasuda, S., Ho, M., Nishijima, M., Brown, R. H., Holleran, W. M., and Hanada, K. (2002) Hereditary sensory neuropathy type 1 mutations confer dominant negative effects on serine palmitoyltransferase, critical for sphingolipid synthesis. *J. Clin. Invest.* 110, 1301–8

60. Gable, K., Han, G., Monaghan, E., Bacikova, D., Natarajan, M., Williams, R., and Dunn, T. M. (2002) Mutations in the yeast LCB1 and LCB2 genes, including those corresponding to the hereditary sensory neuropathy type I mutations, dominantly inactivate serine palmitoyltransferase. *J. Biol. Chem.* 277, 10194–200

61. Zitomer, N. C., Mitchell, T., Voss, K. a, Bondy, G. S., Pruett, S. T., Garnier-Amblard, E. C., Liebeskind, L. S., Park, H., Wang, E., Sullards, M. C., Merrill, A. H., and Riley, R. T. (2009) Ceramide synthase inhibition by fumonisin B1 causes accumulation of 1-deoxysphinganine: a novel category of bioactive 1-deoxysphingoid bases and 1-deoxydihydroceramides biosynthesized by mammalian cell lines and animals. *J. Biol. Chem.* 284, 4786–95

62. Penno, A., Reilly, M. M., Houlden, H., Laurá, M., Rentsch, K., Niederkofler, V., Stoeckli, E. T., Nicholson, G., Eichler, F., Brown, R. H., von Eckardstein, A., and

Hornemann, T. (2010) Hereditary sensory neuropathy type 1 is caused by the accumulation of two neurotoxic sphingolipids. *J. Biol. Chem.* 285, 11178–87

63. Baird, R. D., Kitzen, J., Clarke, P. a, Planting, A., Reade, S., Reid, A., Welsh, L., López Lázaro, L., de las Heras, B., Judson, I. R., Kaye, S. B., Eskens, F., Workman, P., deBono, J. S., and Verweij, J. (2009) Phase I safety, pharmacokinetic, and pharmacogenomic trial of ES-285, a novel marine cytotoxic agent, administered to adult patients with advanced solid tumors. *Mol. Cancer Ther.* 8, 1430–7

64. Schöffski, P., Dumez, H., Ruijter, R., Miguel-Lillo, B., Soto-Matos, a, Alfaro, V., and Giaccone, G. (2011) Spisulosine (ES-285) given as a weekly three-hour intravenous infusion: results of a phase I dose-escalating study in patients with advanced solid malignancies. *Cancer Chemother. Pharmacol.* 68, 1397–403

65. Garofalo, K., Penno, A., and Schmidt, B. (2011) Oral l-serine supplementation reduces production of neurotoxic deoxysphingolipids in mice and humans with hereditary sensory autonomic neuropathy type 1. *J. Clin. …*. 10.1172/JCI57549.)

66. Bertea, M., Rütti, M. F., Othman, A., Marti-Jaun, J., Hersberger, M., von Eckardstein, A., and Hornemann, T. (2010) Deoxysphingoid bases as plasma markers in diabetes mellitus. *Lipids Health Dis.* 9, 84

67. Othman, A., Rütti, M. F., Ernst, D., Saely, C. H., Rein, P., Drexel, H., Porretta-Serapiglia, C., Lauria, G., Bianchi, R., von Eckardstein, A., and Hornemann, T. (2012) Plasma deoxysphingolipids: a novel class of biomarkers for the metabolic syndrome? *Diabetologia*. 55, 421–31

2 Mutations at the amino acid position S331 of SPTLC1 are associated with a distinct syndromic phenotype

Published in European Journal of Medical Genetics Volume 56, Issue 5, May 2013, Pages 266–269

Michaela Auer-Grumbach[1, 2*], Heiko Bode[3, 4], Thomas Pieber[1], Maria Schabhüttl[1], Dirk Fischer[5, 6], Rainer Seidl[7], Elisabeth Graf[8], Thomas Wieland[8], Gerda Vacariu[2], Franz Grill[2], Vincent Timmerman[9], Tim Strom[8, 10], Thorsten Hornemann[3, 4,]

[1] Department of Internal Medicine, Division of Endocrinology and Metabolism, Medical University of Graz, Graz, Austria

[2] Orthopedical Hospital Speising, Vienna, Austria

[3] Institute of Physiology and Zürich, Center for Integrative Human Physiology (ZIHP), University of Zürich, Zürich, Switzerland

[4]Institute for Clinical Chemistry, University Hospital Zürich, Zürich, Switzerland

[5]Department of Neurology, University of Basel Hospital, Basel, Switzerland

[6]Department of Neuropaediatrics, University of Basel Children's Hospital, Basel, Switzerland

[7] Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, Vienna, Austria

[8]Institute of Human Genetics, Helmholtz Zentrum München - German Research Center for Environmental Health, Neuherberg, Germany

[9] VIB - Department of Molecular Genetics, Peripheral Neuropathy Group, University of Antwerp - CDE, Antwerpen, Belgium

[10]Institute for Human Genetics, Technical University Munich, Munich, Germany

This Chapter was published in European Journal of Medical Genetics Volume 56, Issue 5, May 2013, Pages 266–269 by Michaela Auer-Grumbach. Text, figures and pictures have been reproduced from this original publication.

My contribution to this publication started with the cloning of the new p.S331Y mutation into our expression system. I transfected this new mutation into HEK293 cells and generated the stable mutant-expressing cell line. I measured *de novo* production of C18SA and 1-deoxySA (DoxSA) by those cells under FB1 treatment and also the concentrations of 1-deoxySA and 1-deoxySO (DoxSO) in the plasma of the patient and family controls. These data are presented in Figure 2 a and b.

The plasma from this new patient (SPTLC1 p.S331Y) and the family controls was kindly provided by the neurologist in charge, Michaela Auer-Grumbach. The plasma samples cntrl 1 and cntrl 2 were obtained from healthy members of our lab.

2.1 Abstract

Mutations in the serine palmitoyltransferase subunit 1 (SPTLC1) gene are the most common cause of hereditary sensory neuropathy type 1 (HSN1). Here we report the clinical and molecular consequences of a particular mutation (p.S331Y) in SPTLC1 affecting a patient with severe, diffuse muscle wasting and hypotonia, prominent distal sensory disturbances, joint hypermobility, bilateral cataracts and considerable growth retardation. Normal plasma sphingolipids were unchanged but 1-deoxy-sphingolipids were significantly elevated. In contrast to other HSN patients reported so far, our findings strongly indicate that mutations at amino acid position Ser331 of the SPTLC1 gene lead to a distinct syndrome.

2.2 Introduction

Hereditary sensory neuropathies (HSN) are clinically and genetically heterogeneous disorders of autosomal dominant or autosomal recessive inheritance characterized by axonal atrophy and degeneration, predominantly affecting the sensory neurons (1-3). Hall- mark features of the dominantly inherited variant subclassified as HSN type 1 (HSN1) comprise severe distal sensory loss, painless injuries, skin ulcers and frequent bone infections that sometimes necessitate amputations of toes or feet (1-3). Disease onset is usually in early adulthood. Variable distal muscle weakness and wasting and lancinating pain is also often observed. With disease progression the hands may become involved similarly (1-3). Mutations in the serine palmitoyltransferase, long chain base subunit 1 (SPTLC1) gene are themost frequent cause of HSN1[4,5]. SPTLC1 encodes one of the three subunits of serine palmitoyltransferase (SPT), which catalyzes the first step in the de-novo synthesis of sphingolipids which is the condensation of L-serine and palmitoyl-coenzyme A. Under certain conditions SPT shows a shift from its canonical substrate L-serine to the alternative substrates L-alanine and glycine which leads to the formation of an atypical class of 1-deoxy-sphingolipids (1-deoxySL). Low levels of 1-deoxySLs are typically present in plasma of healthy individuals. Pathologically elevated 1-deoxySLs have been found in transgenic HSN1 mouse models and in HSN1 patients carrying different SPTLC1mutationsbut also in individuals with the metabolic syndrome and diabetes (6-8). The 1-deoxySLs show pronounced neurotoxic effects in vitro and may be disease causing in HSN1 but are possibly also involved in the pathology of the diabetic neuropathy. The observation that a high dose L-serine supplementation lowers plasma 1-deoxySL levels in HSN1 patients and in the transgenic mouse model has encouraged our hopes for a first treatment of this ulcero-mutilating disorder. This emphasizes the importance of an early and accurate genetic diagnosis ofHSN1patients (9). Here we report a novel SPTLC1 mutation in a female patient exhibiting an unusually severe and complicated phenotype. We show that mutations at the

particular amino acid position Serine 331 (S331) are associated with a distinct syndromic phenotype in comparison to previously reported mutations in HSN1.

2.3 Patient and methods

The female proband was the first child of non-consanguineous and healthy parents. Pregnancy was normal but she was born by cesarean delivery three weeks early. Motor milestones during the first years were normal but height and weight always ranked low in percentile or they were even below the lower limit. At 4 years of age a strange gait, frequent falls and moderate hand tremor were noted. Sensory disturbances were initially mild but progressed with dis- ease. Considerable *pes cavus* foot deformity necessitated triple arthrodesis at age 5. Subsequently, the diagnosis of hereditary motor and sensory neuropathy (HMSN) was made. Disease progression was rapid and soon muscle weakness and wasting affected upper limbs, but also proximal limb and trunk muscles thus leading to severe scoliosis, respiratory problems and wheelchair dependence at age 14. In addition, there was prominent growth retardation and delayed puberty whereas intellectual development was normal. At age 13, bilateral cataracts were diagnosed and surgically treated. On examination at age 12 years there was general muscle hypotrophy and hypotonia with pronounced weakness in the distal muscles of the upper and lower limbs (Fig. 1). There were prominent sensory disturbances which were pronounced in the feet and affected all qualities except for the vibration sense which remained completely preserved. At the toes scars after burns due to reduced prominent pain and temperature sensation were evident (Fig. 1, arrow). Hypermobility of the joints, bilateral hand tremor and fasciculations, which were most prominent in the tongue, were observed (Fig. 1). Tendon reflexes were brisk but the Babinski sign was negative. Further evaluation included nerve conduction studies (NCS), magnetic resonance imaging (MRI) studies and molecular genetic testing which followed standard methods. Whole exome sequencing was performed on a Genome Analyzer HiSeq 2000 system (Illumina, San Diego, CA.). Methods and parameters were used as shown previously (10). The plasma sphingoid base profile and cellular SPT activity was analyzed as described previously (11). The study was approved by the local ethical committee.

Figure 1 Clinical features of the patient. Diffuse muscle hypotrophy and hypermobility of joints in the patient carrying the p.S331Y SPTLC1 mutation. Note scars at the toes (arrow) after burns due to reduced pain and temperature sensation.

2.4 Results

Nerve conduction velocities in the upper limbs were within the intermediate range (median motor: 32 m/s, median sensory: 39 m/s, SNAP: 16.7 mV) and compound motor action potentials (CMAP: 2.5 mV, peak-peak) were reduced. There was no response for motor or sensory nerves in the lower limbs. Based on these results the patient was initially classified as HMSN type II. MRI of the brain and spinal cord was normal at the age of 9 years. Endocrinological examination of the patient did not reveal any additional disturbances. After exclusion of mutations in all common HMSN and SCA (spino-cerebellar ataxia) genes, whole exome sequencing was carried out. Thereby, a heterozygous missense change in SPTLC1 (c.992G- > T; p.S331Y) was detected and confirmed by Sanger re- sequencing. The parents were normal on neurological and on electrophysiological examination and this change was absent in both of them. Also, no sequence variation at aa position S331 was found in 1969 individuals from our in-house exomes. Subsequently,we measured the 1-deoxySL levels in the patients' plasma which were found to be significantly elevated (Fig. 2a). Increased 1-deoxySL formation was also confirmed in HEK293 cells expressing the p.S331F and p.S331Y mutant (Fig. 2b). In comparison 1-deoxySL formation was lower in the p.S331Y than in the previously reported p.S331F mutant. Canonical serine activity was reduced by 60% in both mutants.

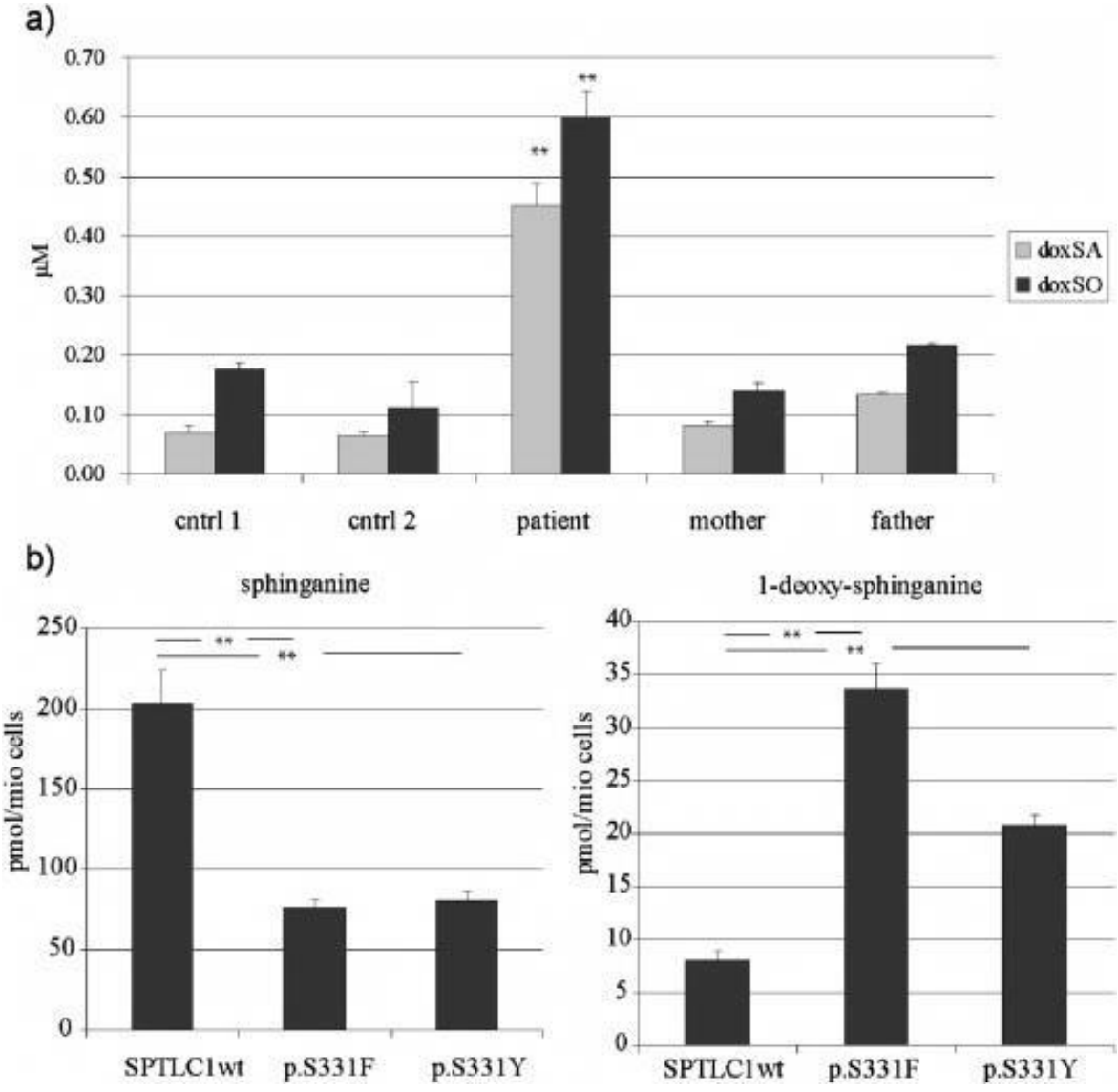


Figure 2 a) 1-DeoxySL levels in plasma samples of the patient carrying the p.S331Y mutation, family members and controls. The levels of the 1-deoxy-sphiglipids were significantly increased in the plasma of the S331Y patient but not in the plasma of the unaffected parents or unrelated healthy controls. Total sphingolipid levels were not different between patients and controls (data not shown).
b) *De-novo* generation of sphinganine and 1-deoxy-sphinganine. HEK293 cells expressing either the S133F or S331Y mutant show a significantly reduced canonical SPT activity and a significantly increased formation of 1-deoxy-sphinganine in comparison to SPTLC1wt expressing cells. The 1-deoxy-sphinganine formation was about 30% lower for the S331Y than for the S331F mutation ($p < 0.05$).**

2.5 Discussion

To date, six disease causing mutations in SPTLC1 (p.C133W, p.C133Y, p.C133R, p.V144D, p.S331F, p.A352V) have been described (5). SPLTC1 mutations at positions p.C133, p.C144 and p.A352 result in the typical HSN1 phenotype (5,12). In contrast, the p.S331F mutation, which was reported in two patients only, occurred *de-novo* and was associated with an extraordinary severe and complicated phenotype. One of these patients was initially diagnosed as early onset HMSN type II due to muscle weakness and hypotrophy in addition to prominent sensory disturbances, bone fractures and osteomyelitis. Notably, this patient also developed juvenile cataract at age of 9 years, complete retinal detachment at 10 and repetitive corneal ulceration and keratitis with poor corneal healing (13). An even more complex congenital phenotype with severe growth retardation, global amyotrophy, hypotonia, joint hyperlaxity, vocal cord paralysis, bilateral cataract, mild mental retardation, microcephaly and respiratory problems was described in the second patient (5). The pathogenicity of the p.S331F mutation was confirmed by functional studies: in vitro a reduction of SPT activity was shown and in plasma samples of both patients increased levels of 1-deoxySLs were detected (9). Nevertheless, it remained uncertain whether these additional features were also related to the p.S331F mutation (12). The patient reported here carries a novel mutation in SPTLC1 leading to a change of serine to tyrosine at aa position 331 which also results in a similar unusually severe and complicated phenotype. The core phenotype of the three patients described so far carrying an S331 SPTLC1 mutation resembles early onset HMSN. The additional features highly enlarge the phenotype of HSN1 but are rather unique between the three patients (Table 1) thus proposing the existence of a distinct and severe syndrome associated with this particular mutation. Since oral L-Serine supplementation has been suggested to be a future treatment option, it will be most important to achieve a quick diagnosis by early screening of exon 11 of SPTLC1 in patients exhibiting this distinct phenotype.

Reported associated features as part of the S331-*SPTLC1* syndrome	Huehne et al, 2008 (13) (p.S331F)	Rotthier et al, 2009 (5) (p.S331F)	This study (p.S331Y)
Diffuse muscle hypotrophy	Yes	Yes	Yes
Growth retardation	nm	Yes	Yes
Motor and sensory neuropathy	Yes	Yes	Yes
Foot ulcers, amputations, combustions and/or burns	Yes	Yes	Yes
Juvenile cataracts	Yes	Yes	Yes
Mental retardation	nm	Yes	No
Joint hypermobility	nm	Yes	Yes
Vocal cord paralysis	nm	Yes	No
Tremor	nm	nm	Yes
Fasciculations	nm	nm	Yes
Other ocular manifestations	Yes	nm	No
Respiratory problems	nm	Yes	Yes

Table 1 Core features of the syndromic phenotype produced by the S331 SPTLC1 mutation. Features present in at least two patients are highlighted in bold (nm: not mentioned).

2.6 Acknowledgement

We would like to thank the patient and her parents for participation in this study. This work was supported by the Austrian Science Fund (FWF P23223-B19).

2.7 References for Chapter 2:

(1) A. Rotthier, J. Baets, V. Timmerman, K. Janssens, Mechanisms of disease in hereditary sensory and autonomic neuropathies,Nat. Rev.Neurol. 8 (2) (2012)73e85.

(2) M. Auer-Grumbach, P. De Jonghe, K. Verhoeven, V. Timmerman, K. Wagner, H.P. Hartung, G.A. Nicholson, Autosomal dominant inherited neuropathies with prominent sensory loss and mutilations, Arch. Neurol. 60 (3) (2003) 329e334.

(3) P.J. Dyck, Neuronal Atrophy and Degeneration Predominantly Affecting Peripheral Sensory and Autonomic Neurons. Peripheral Neuropathy, third ed., WB Saunders Company, 1993, pp. 1065e1093.

(4) J.L. Dawkins, D.J. Hulme, S.B. Brahmbhatt, M. Auer-Grumbach, G.A. Nicholson, Mutations in SPTLC1, encoding serine palmitoyltransferase, long chain subunit-1 cause hereditary sensory neuropathy type I, Nat. Genet. 27 (3) (2001) 309e312.

(5) A. Rotthier, J. Baets, E. De Vriendt, A. Jacobs, M. Auer-Grumbach, N. Lévy, N. Bonello-Palot, S.S. Kilic, J. Weis, A. Nascimento, M. Swinkels, M.C. Kruyt, A. Jordanova, P. De Jonghe, V. Timmerman, Genes for hereditary sensory and autonomic neuropathies: a genotype-phenotype correlation, Brain 132 (2009) 2699e2711.

(6) M. Bertea, M.F. Rütti, A. Othman, J. Marti-Jaun, M. Hersberger, A. von Eckardstein, T. Hornemann, Deoxysphingoid bases as plasma markers in diabetes mellitus, Lipids Health Dis. 9 (2010) 84.

(7) A. Othman, M.F. Rütti, D. Ernst, C.H. Saely, P. Rein, H. Drexel, C. Porretta-Serapiglia, G. Lauria, R. Bianchi, A. von Eckardstein, T. Hornemann, Plasma deoxysphingolipids: a novel class of biomarkers for the metabolic syndrome? Diabetologia 55 (2) (2012) 421e431.

(8) R. Wang-Sattler, Z. Yu, C. Herder, A.C. Messias, A. Floegel, Y. He, K. Heim, M. Campillos, C. Holzapfel, B. Thorand, H. Grallert, T. Xu, E. Bader, C. Huth, K. Mittelstrass, A. Döring, C. Meisinger, C. Gieger, C. Prehn, W. Roemisch- Margl, M. Carstensen, L. Xie, H. Yamanaka-Okumura, G. Xing, U. Ceglarek, J. Thiery, G. Giani, H. Lickert, X. Lin, Y. Li, H. Boeing, H.G. Joost, M.H. de Angelis, W. Rathmann, K. Suhre, H. Prokisch, A. Peters, T. Meitinger, M. Roden, H.E. Wichmann, T. Pischon, J. Adamski, T. Illig, Novel biomarkers for prediabetes identified by metabolomics, Mol. Syst. Biol. 8 (2012) 615.

(9) K. Garofalo, A. Penno, B.P. Schmidt, H.J. Lee, M.P. Frosch, A. von Eckardstein, R.H. Brown, T. Hornemann, F.S. Eichler, Oral L-serine supplementation reduces production of neurotoxic deoxysphingolipids in mice and humans with hereditary sensory autonomic neuropathy type 1, J. Clin. Invest. 121 (12) (2011) 4735e4745.

(10) C. Beetz, T.R. Pieber, N. Hertel, M. Schabhüttl, C. Fischer, S. Trajanoski, E. Graf, S. Keiner, I. Kurth, T. Wieland, R.E. Varga, V. Timmerman, M.M. Reilly, T.M. Strom, M. Auer-Grumbach, Exome sequencing identifies a REEP1 mutation involved in distal hereditary motor neuropathy type V, Am. J. Hum. Genet. 91 (1) (2012) 139e145.

(11) A. Penno, M.M. Reilly, H. Houlden, M. Laurá, K. Rentsch, V. Niederkofler, E.T. Stoeckli, G. Nicholson, F. Eichler, R.H. Brown Jr., A. von Eckardstein, T. Hornemann, Hereditary sensory neuropathy type 1 is caused by the accumulation of two neurotoxic sphingolipids, J. Biol. Chem. 285 (15) (2010) 11178e11187.

(12) A. Rotthier, A. Penno, B. Rautenstrauß, M. Auer-Grumbach, G.M. Stettner, B. Asselbergh, K. Van Hoof, H. Sticht, N. Lévy, V. Timmerman, T. Hornemann, K. Janssens, Characterization of two mutations in the SPTLC1 subunit of serine palmitoyltransferase associated with hereditary sensory and autonomic neuropathy type I, Hum. Mutat. 32 (6) (2011) E2211eE2225.

(13) K. Huehne, C. Zweier, K. Raab, S. Odent, M. Bonnaure-Mallet, J.L. Sixou, P. Landrieu, C. Goizet, J. Sarlangue, M. Baumann, T. Eggermann, A. Rauch, S. Ruppert, G.M. Stettner, B. Rautenstrauss, Novel missense, insertion and deletion mutations in the neurotrophic tyrosine kinase receptor type I gene (NTRK1) associated with congenital insensitivity to pain with anhidrosis, Neuromuscul. Disord. 18 (2) (2008) 159e166.

3 Treating severe hereditary sensory and autonomic neuropathy type 1 caused by a mutation p.S331F in SPTLC1

An experimental serine therapy in a German boy verifies serine as an effective treatment even for severe cases of HSAN1 and establishes an additional biomarker for mutations at Ser331.

Heiko Bode, Bernd Rautenstrauss, Ekkehard Wilichowski, Thorsten Hornemann

Unpublished manuscript

This Chapter has not been published in a peer reviewed journal. The clinical description of the patient and, the improvements of the health status and the progression of the pathology under serine therapy are based on communications with the neurologist in charge Ekkehard Wilichowski and the mother of the patient. Pictures and plasma of the patient and his family were kindly provided by Ekkehard Wilichowski. The experimental therapy with L-serine was and still is conducted under clinical supervision of the neurologists in charge.

I extracted the sphingolipids from the plasma samples and measured, and quantified the different sphingolipid backbones, as shown in Figure 3-2 -Figure 3-6.

3.1 Abstract:

Serine palmitoyltransferase (SPT) catalyzes the first and rate limiting step of the sphingolipid *de novo* synthesis, the condensation of the amino acid L-serine and the activated fatty acid palmitoyl-CoA. Mutations in SPT are known to cause the rare hereditary sensory and autonomic neuropathy type 1 (HSAN1). HSAN1-causing mutations in SPT increase its affinity for the alternative substrates L-alanine and glycine, giving rise to the generation of a neurotoxic and atypical class of 1-deoxysphingolipids (1-deoxySLs). Two of the first identified and also most frequently reported HSAN1 mutations affect the cysteine residue at p.133 of serine palmitolytransferase long chain base subunit 1 (SPTLC1 p.C133W and SPTLC1 p.C133Y). Supplementation with high concentrations of L-serine was shown to effectively lower the concentrations of 1-deoxySLs in cells, plasma and nerves of mice bearing the p.C133W mutation, and also in the plasma of patients with the p.C133Y mutation. Compared to these two mutations, two more recently described mutations at Ser331 of SPTLC1 (p.S331F and p.S331Y) were shown to cause a particularly severe phenotype, characterized by early onset, pronounced diffuse muscle wasting, and additional symptoms like anhydrosis, and impairment of the visual and respiratory system. Typical HSAN1 symptoms like sensory loss, poor wound healing and development of ulcers are also present in Ser331 patients and tend to aggravate faster than in the classical HSAN1 mutations at Cys133.

Here we report the case of a German boy carrying the p.S331F mutation in SPTLC1, who participated in an experimental serine therapy study. The trial started in 2011 and is still ongoing. The plasma sphingolipid profile of the patient was measured over a period of 22 months. We observed a significant reduction of plasma 1-deoxySLs in response to L-serine treatment. Clinical examinations and patient testimonies showed marked improvements of their physical state in response to the therapy.

Besides highly increased 1-deoxySL levels, recent results showed that mutations in S331 are also associated with a significantly increased formation of C20 sphingoid bases. Elevated C20 sphingoid bases were found in S331F-expressing cells and in patient plasma. Elevation of C20 sphingoid bases was not associated with any of the other HSAN1 mutations. The increased formation of C20 sphingoid bases therefore is a specific feature of the S331 mutation and represents an additional biomarker for this more severe form of HSAN1.

3.2 Introduction:

Hereditary sensory and autonomic neuropathies (HSANs) are a group of clinically and genetically heterogeneous diseases of the peripheral nervous system. HSANs are associated with mutations in thirteen different genes and represent up to nine clinically different neuropathies (1).

The hereditary sensory and autonomic neuropathy type 1 (HSAN1) represents an exception within this large family because of its autosomal dominant mode of inheritance, contrary to the autosomal recessive inheritance in all other types of HSAN. It can be further discriminated from subtypes 2-7 by its late onset between the 2nd and 5th decades, the slow progression of the neuropathic symptoms, and the characteristically observed tissue complications like plantar ulcers which in HSAN1 predominantly affect the lower limbs (2, 3).

Mutations in subunit 1 of the multimeric SPT complex were first linked to HSAN1 in 2001 (4, 5). Until today, seven mutations in serine palmitoyltransferase long chain base subunit 1 (SPTLC1 p.C133W, p.C133Y, p.C133R, p.V144D, p.A352V, p.S331F and p.S331Y) and another six in SPT long chain base subunit 2 (SPTLC2 p.A182P, p.V359M, p.G382V, p.T409M, p.I504F and p.S384F) have been reported to cause the two most frequent and identical phenotypes of HSAN1 (SPTLC1→HSAN1A OMIM#162400 and SPTLC2→HSAN1C OMIM#613640) (4–11).

The canonical function of SPT is the condensation of the activated fatty acid palmitoyl-CoA and the amino acid L-serine, the first and rate limiting step of sphingolipid (SL) *de novo* synthesis. HSAN1 mutations were shown to cause substrate promiscuity of SPT, resulting in an increased activity with the alternative substrates L-alanine and glycine. Condensation of palmitoyl CoA with L-alanine thereby leads to the formation of 1-deoxysphinganine (1-deoxySA), while the use of glycine generates 1-desoxymethylsphinganine (1-desoxyMeSa) (12). Elevated 1-deoxySL concentrations were consistently found in the plasma of all HSAN1 patients with mutations in SPT and represent a hallmark and reliable biomarker for HSAN1.

Besides this pronounced genotypic heterogeneity, the severity of the symptoms in HSAN1 is also highly variable. This is reflected by the age of onset, ranging from congenital to more than 50 years, and even the total absence of any symptoms (3, 8), but also by the general progression of the disease and the degree of impairment of nerve conduction velocity or muscle wasting.

Mutations of the serine residue at p.331 in SPTLC1 are associated with an exceptionally severe phenotype (6). Besides the typical HSAN1 symptoms such as loss of pain and temperature sensation in distal limbs, and development of ulcers and osteomyelitis, patients

bearing a mutation at the highly conserved Ser331 present with an early or even congenital onset of symptoms, considerable motor involvement, global hypotrophy, growth and in some cases even mental retardation, development of juvenile cataracts, vocal cord palsy, anhydrosis, and severe respiratory problems (6, 9, 13, 14). Up to now four patients with substitutions of Ser331 in SPTLC1 were reported. An exchange of Ser331 for Phenylalanine (p.S331F) was found in a German boy (patient no. MGZ-48522) (13, 15), a French girl (patient no. CMT-791.01) (9), and a Korean patient who died at age 28 (14). An S331Y exchange was reported in an Austrian girl and is associated with an equally severe phenotype (6).

As there is no cure for HSAN1 available yet, medical intervention was and is limited to symptomatic interventions like ortheses to stabilize ankle joints, optimized orthopedic footwear, and wound care to prevent and treat small injuries and skin lesions and to prevent their progression to chronic ulcers and osteomyelitis. In addition, modern pain medication helps to relieve the patients from neuropathic pain but fails to cure the underlying cause.

Studies in cell culture and in mice expressing the p.C133W mutation showed that *de novo* formation of the atypical 1-deoxySLs can be effectively prevented by oral supplementation with high doses of L-serine. These findings were verified in a first pilot study in HSAN1 patients bearing the p.C133Y variant of SPTLC1 (16). Fourteen patients received either a low (200mg/kg bodyweight) or high dose (400mg/kg) of L-serine for 10 weeks. Plasma sphingolipid profiles were repeatedly measured and a significant decrease of 1-deoxySL levels was observed in both groups within six weeks. During the washout phase after ten weeks of serine supplementation, the levels of 1-deoxySLs started to rise again. Although neurological improvements were not systematically assessed within the 12 weeks of the study, several patients reported subjective improvements such as increased sensibility and tingling feeling in the hands, improvements in skin robustness, and nail and hair growth (16).

Here we report the case of a German boy with a spontaneous SPTLC1 p.S331F mutation who presents severe form of HSAN1 with a very early onset. Starting in 2011 the patient participated in an experimental serine therapy study under constant medical supervision. Here we report the course of this therapy over 22 months, together with the improvements and aggravations of neuropathic symptoms.

3.3 The patient:

The hereby reported experimental serine therapy was conducted with a male German HSAN1 patient. The patient was born in May 1994 as the second of four children of non-consanguineous and unaffected parents.

3.3.1 Genetic background and diagnosis

The patient (MGZ-48522) was first reported in 2008 (15) and initially diagnosed with HSAN4 (also known as congenital insensitivity to pain with anhydrosis, CIPA). This early diagnosis was based on the presence of HSAN4-specific symptoms like, disturbed thermal regulation, total absence of pain sensation in lower limbs, and onset of first symptoms in early childhood (2, 17). However, other typical symptoms of HSAN4 like mental retardation and self-mutilating behavior were absent in the patient and several other clinical symptoms like distal muscle weakness, hypotrophy, joint contractures and the measured neurophysiological parameters complied rather with a HMSNII or HSAN phenotype (15). Resequencing of the patient (MGZ-48522) in 2011 finally revealed a heterozygous *de novo* mutation in SPTLC1 c.992C>T (p.S331F) as the genetic cause of his peripheral neuropathy. This mutation was not found in unaffected family members. Identification of this *de novo* mutation in SPTLC1 finally confirmed the assumption the patient might suffer from HSAN1 rather than HSAN4 (13).

3.3.2 Clinical description of the patient

The patient presented first motoric symptoms at 18 months. He could not sit for a long time, and a waddling gait and frequent falls were noticed. Gait abnormalities were treated by physiotherapy. Difficulties in climbing stairs, but also climbing and jumping in general followed shortly afterwards. At three years, global hypotrophy and weakness of distal musculature were diagnosed together with finger tremor and pes cavus. Walking distance was limited to 500-600m and difficulties with stair climbing had aggravated. At six years he got ortheses, and the use of a wheelchair became indispensable for longer walking distances. Fine motoric skills were also impaired and the use of a writing computer became inevitable at eight years. At nine years, contractures of finger-, hip-, knee- and elbow joints developed, and from his 14th year on the patient permanently depended on the use of a wheelchair for locomotion.

Sensory and autonomous impairments were noticed starting at the age of three. In the beginning, the patient presented with allodynia and also rarely with hypalgesia. At five years, pain sensations had decreased predominantly in lower limbs. Subsequently, the first painless ulcerating wounds developed and even a fracture of the metatarsus remained unnoticed. At

the age of eleven another event of hypalgesia at the lower limbs was observed. Two years later the touch sensation at the lower limbs was decreased and temperature perception of the skin was limited to the small gluteal area.

Since his early childhood the patient presented with very slow healing of rhagades and ulcerations which frequently formed after unnoticed burns, injuries, or after falls. Starting from twelve years on, the patient had permanent wounds on his hands and had to wear gloves all the time to prevent the formation of new wounds. In 2007, spondilocystis, a compression fracture, and osteomyelitis of the upper ankle joint were diagnosed. One year later a pressure sore, caused by the orthesis, got infected with *Staphylococcus aureus* and developed into a purulent phlegmon.

Already in his early childhood the patient started to develop cataracts, making bilateral lensectomy necessary at the age of nine years. One year later retinal detachment occurred and was followed by recidivating conjunctivitis, and corneal clouding.

At six years the patient showed first symptoms affecting the respiratory system as well. He had respiratory dropouts during the night and needed nocturnal intermittent self-ventilation. At nine years he encountered an acute event of dyspnea with cyanosis but without hypercapnia. Frequent infections of the upper respiratory tract especially during winter time made the use of a cough assist necessary for the first time at the age of twelve and inevitable at fourteen.

Motoric nerve conduction velocity (NCV) was 22-23m/sec in *N. peronaeus* at the age of three and no longer recordable three years later. Sensory NCV was not recordable in *N. suralis* and was 38m/sec in *N. medianus* at the age of three. At six years NCV was reduced to 22m/sec and five years later it was no longer recordable in *N. medianus*.

Muscle biopsy revealed substantial variability of fiber diameter and atrophies affecting single and grouped muscle fibers. Biopsy of *N. suralis* in 2007 confirmed the diagnosis of peripheral neuropathy and revealed an almost complete loss of myelinated fibers with signs of axonal damage.

At the study baseline in October 2011, the 17-year old patient weighed 25.4kg at a height of 1.49 m.

Figure 3-1 Pictures of patient MGZ-48522 aged 17 before the therapy. Contractures of the wrists and hypermobility of joints are obvious. Poor wound healing, delicate skin, and absence of pain perception had caused several chronic and slowly healing wounds on the hands and fingers. Pictures provided by Ekkehard Wilichowski.

3.4 Methods:

3.4.1 Sphingolipid extraction from human plasma

One hundred microliters of human plasma (EDTA) were aliquoted into a 2ml microreaction tube and 500µl MeOH (Honeywell), including the deuterated internal standards d7-SA and d7-SO (200pmol each, Avanti Polar lipds, Alabaster CA) were added. Lipids were extracted for 1h under constant agitation at 1400rpm and 37°C on an Eppendorf Thermomixer Comfort (Eppendorf, Hamburg, Germany). Precipitated proteins were removed by centrifugation for 5min at 16100rcf and 22°C in an Eppendorf 5415 table-top centrifuge (Eppendorf, Hamburg, Germany). Five hundred microliters of the supernatant were transferred to fresh tubes and 75µl of concentrated HCl (32%, Sigma) were added. The extracted lipids were hydrolyzed overnight (for at least 12 hours) at 65°C. Acid hydrolysis was stopped by addition of 100µl KOH [10M], and sphingolipid extraction and purification was started with the addition of 125µl CHCl3. Separation of the organic and inorganic phases was initiated by addition of another 500µl CHCl3, followed by 100µl NH4OH [2N] and finally 500 µl alkaline H2O. Samples were mixed by vigorous vortexing after the addition of each solvent. Phase separation was leveraged by centrifugation at 16100rcf and 22°C for another 5min. The upper polar phase was removed using a Pasteur pipette and vacuum pump (BVC 21 NT Vario, Vacuubrand, Theilingen, Switzerland). The chloroform phase was washed two more times with 1ml alkaline H2O. The remaining CHCl3 phase was evaporated under constant N2-flow in Techne Sample Concentrator (Bibby Scientific Ltd, Staffordshire, UK) for ~20min. Dried lipids were stored at -20°C.

3.4.2 Derivatization and LC-MS analysis:

Dried lipids were redissolved in 75µl of derivatization mix (56.7% MeOH, 33.3% EtOH, 10% H2O) and derrivatized with 5µl of OPA working solution (990µl boric acid [3%] + 10µl O-phthalaldehyde [50mg/ml in EtOH] + 0.5µl 2-Mercaptoethanol).

Samples were separated on a reverse phase C18 column (Uptispere 120 Å, 5µm, 125x2 mm, Interchim, France) at a flowrate of 400µl/min. Mass spectra were recorded in full scan positive ion mode by a triple quad detector (TSQ quantum ultra, Thermo scientific).

Peaks were analyzed and the areas under the peaks were calculated using Xcalibur (Thermo scientific).

3.5 Results:

3.5.1 The Therapy:

Starting from 11.10.2011 the patient participated in an experimental serine therapy trial. At baseline, plasma samples were drawn and the sphingoid base profiles of the patient and three unaffected family members were measured. As shown in Figure 3-2, the concentrations of the most abundant C18-based sphingolipids were not significantly changed between the patient and the family controls, whereas the concentrations of the minor C20-based sphingolipids and the atypical 1-deoxysphingolipids were both significantly elevated in the plasma of the patient.

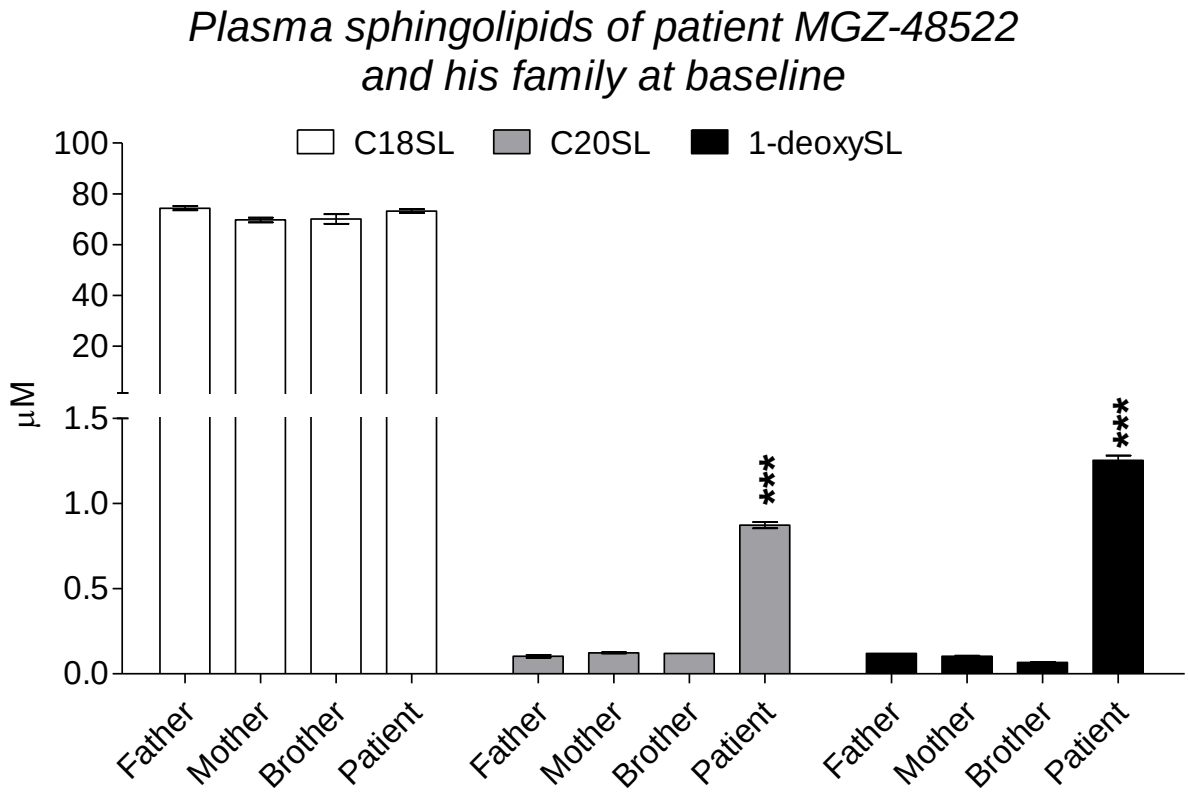


Figure 3-2 Concentrations of sphingolipids in plasma of the patient and three unaffected members of his family. Sphingolipids (SLs) were extracted from 100µl of EDTA plasma, the second fatty acid and head groups were removed by acid and base hydrolysis, and the sphingoid base backbones were measured by LC-MS. Three different classes (C18-based SLs, C20-based SLs, and 1-deoxySLs) of sphingoid bases are shown as the sums of the respective sphingosine and sphinganine backbones (C18SO+C18SA, C20SO+C20SA and 1-deoxySO+1-deoxySA, respectively). Significance of the difference was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001..

We compared the sphingolipid profiles of patient MGZ-48522 not only to the profiles of healthy family controls, but also to other patients bearing other HSAN1 mutations in SPT. We choose the French p.S331F patient (CMT-791.01) and the Austrian p.S331Y patient, expecting to find similar sphingolipid profiles as in MGZ-48522. Furthermore, we included patient samples from the two most frequent HSAN1-causing mutations in SPT, p.C133Y and p.C133W, as well as an unrelated control sample. Figure 3-3 illustrates the mean concentrations of the different sphingoid base backbones, measured by LC-MS. The most abundant sphingoid bases like C16, C17, C18 sphinganine and sphingosine, and C18 sphingadiene presented no obvious differences between the analyzed plasma samples. However, the atypical 1-deoxy sphingoid bases 1-deoxySA and 1-deoxySO were several folds increased in all samples of HSAN1 patients and reached the highest levels in the plasma of the two p.S331F patients. Minimal amounts of 1-desoxymethylsphingosine were detectable only in the plasma of the p.S331F and p.S331Y patients. C20-based sphingolipids were several folds elevated in these three patients as well, while they were the same as the control levels in the plasma of both p.C133 patients.

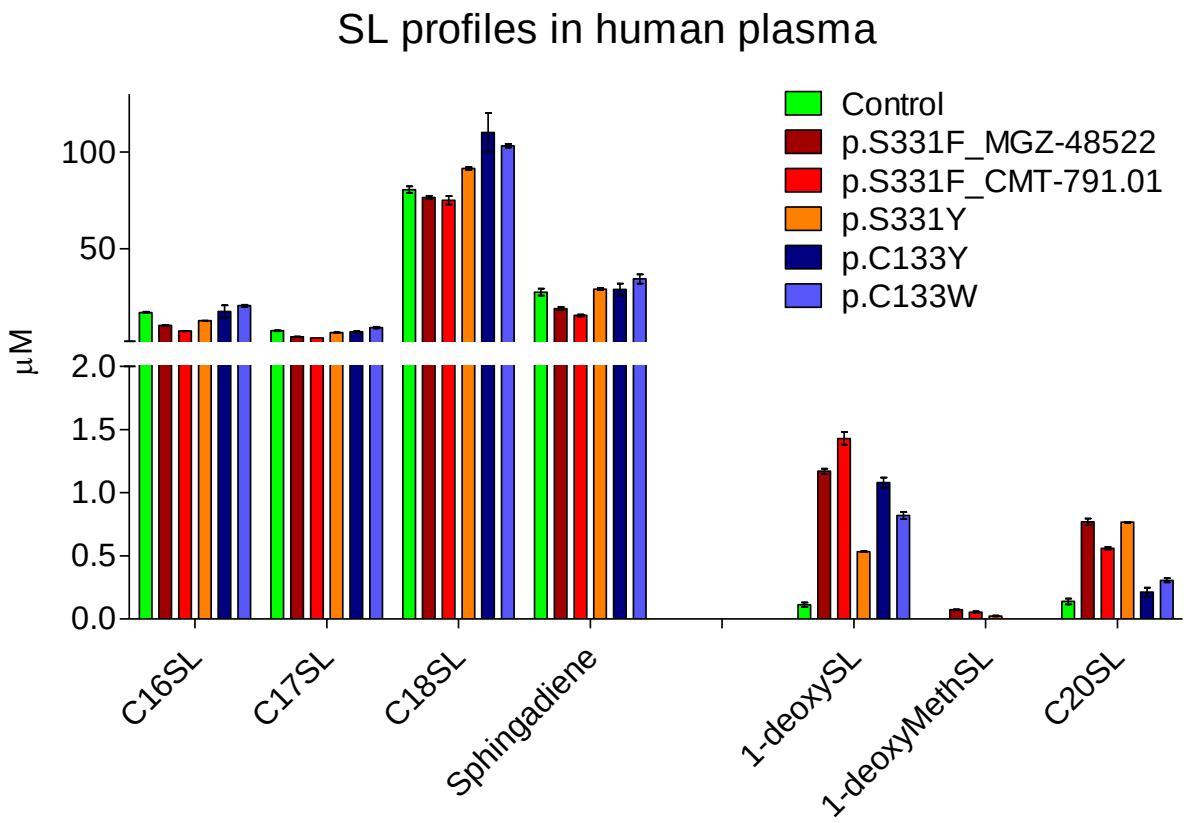


Figure 3-3 Sphingolipid profiles in plasma of healthy individuals and HSAN1 patients. Sphingolipids (SLs) were extracted from human plasma and subjected to acid and base hydrolysis. Free sphingoid bases were analyzed by LC-MS. Results are shown as sums of sphingoid bases with matching carbon chain lengths (e.g. C16 sphinganine + C16 sphingosine or 1-deoxysphinganine + 1-deoxysphingosine, etc.). Sphingadiene is shown as an individual sphingoid base.

3.5.2 Development of SL and 1-deoxySL profiles in patient plasma during the curative trial:

Own preceding studies on the impact of HSAN1-causing mutations on the biochemical properties of SPT had shown that mutations at serine 331 of SPTLC1 cause a global increase in the canonical SPT activity in cells expressing one of the two reported mutations SPTLC1 p.S331F or p.S331Y, respectively (see chapter 2 and 5 of this thesis). Furthermore, increasing concentrations of C18SA and C18SO were measured during the course of the p.C133Y pilot study in human patients under serine supplementation (16). However, we did not detect elevated concentrations of C18-based sphingolipids in plasma of the p.S331Y patient (6), plasma and lymphoblasts of the second p.S331F patient (13), nor baseline plasma of our p.S331F patient.

Nevertheless, considering the observed highly increased canonical activity caused by the p.S331F mutation in our cell culture model and the moderately increasing C18 SLs levels in the p.C133Y patients under L-serine supplementation, we were alarmed that L-serine might result in increased SL *de novo* synthesis in this patient as well. Consequently we expected increased formation of C18-based ceramides which are known for their various pro apoptotic effects on a large variety of cells (see Mullen, Hannun, & Obeid, 2012; Ogretmen & Hannun, 2004 for a review). This would most likely have aggravated the symptoms in the patient or even caused additional problems.

Therefore we initially chose the low dose of L-serine (200mg/kg body weight and day) for the beginning of the therapy. The amino acid L-serine was administered orally in three doses of 1.65g each. Fasted plasma samples for sphingolipid profile measurements were drawn during follow-up examinations after 28 and 42 days to exclude adverse effects of the low dose serine treatment. Until 42 days after start of the supplementation, no detrimental effects on the general health status of the patient were observed; therefore the initial low dosage was increased to the high dose of 400mg/kg body weight and day as already established in the p.C133Y study (16).

During the treatment, routine laboratory tests were conducted. Liver parameters were all in the normal range. Serine concentrations were elevated 2-3-fold over the upper limit in plasma and 1.1-fold in urine.

As shown in Figure 3-2, the levels of C18 SLs were not elevated at baseline (day 0) in comparison to the family controls. After 28 days of serine supplementation with the low dose 200mg/kg, we noticed a significant drop in C18 SL concentration (0.01>p>0.001) (see Figure 3-4). After 42 days under low dose serine supplementation the concentration of C18 SLs had started to increase again. After another 18 days of high dose supplementation with 400mg/kg of L-serine (70 days of therapy in total), the concentration was close to the baseline level again. Subsequently the concentration of C18-based SLs did not show any significant increase or decrease anymore.

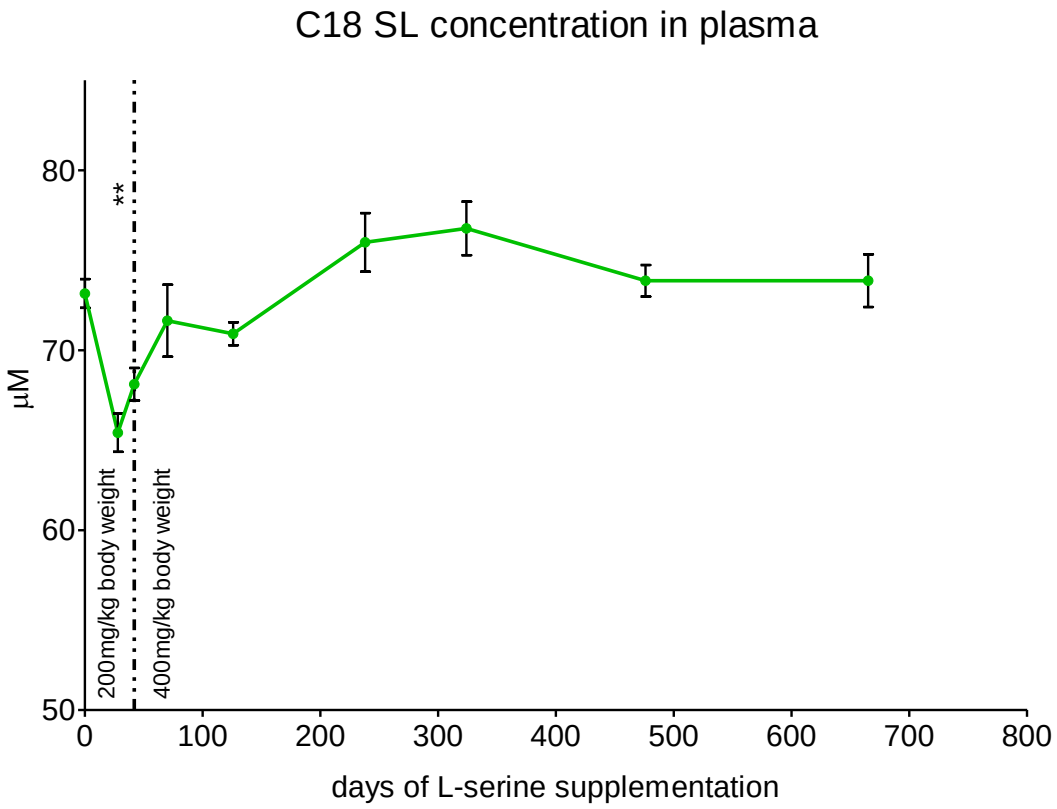


Figure 3-4 Concentrations of normal C18 SLs as measured in plasma of the patient before and during the L-serine therapy. Until day 42 the patient was treated with 200mg of L-serine per kg of body weight which was administered orally. After 42 days the dose was doubled to 400mg/kg of body weight. C18 SL concentrations at baseline showed no deviation from the controls and (considering the full duration of the treatment) did not change significantly during the subsequent L-serine supplementation. Significance of the difference was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with **p < 0.01.

Consistent with our previous results from cells expressing the p.S331F mutation, as well as plasma measurements of other HSAN1 patients with mutations at Ser331, the baseline concentrations of 1-deoxySLs in plasma of the patient were highly elevated compared to the healthy family controls (see Figure 3-2). Oral serine ingestion resulted in a dramatic and fast reduction of the 1-deoxySL concentration to below 50% of the basal levels (see Figure 3-5). The largest decrease thereby occurred within the first seventy days after the start of the trial. For six weeks the therapeutic L-serine dose was limited to 200mg/kg body weight, which was already enough to considerably lower the 1-deoxySL concentration. After increasing the L-serine supplementation to 400mg/kg body weight, 1-deoxySL concentrations kept declining for at least 4 more weeks, but then started to rise again. The second phase of 1-deoxySL decrease under supplementation with 400mg/kg body weight started after 17 weeks, but was much slower and resulted in a continuous reduction of 1-deoxySLs in the plasma of the patient to the nadir of ~45% of the baseline concentration.

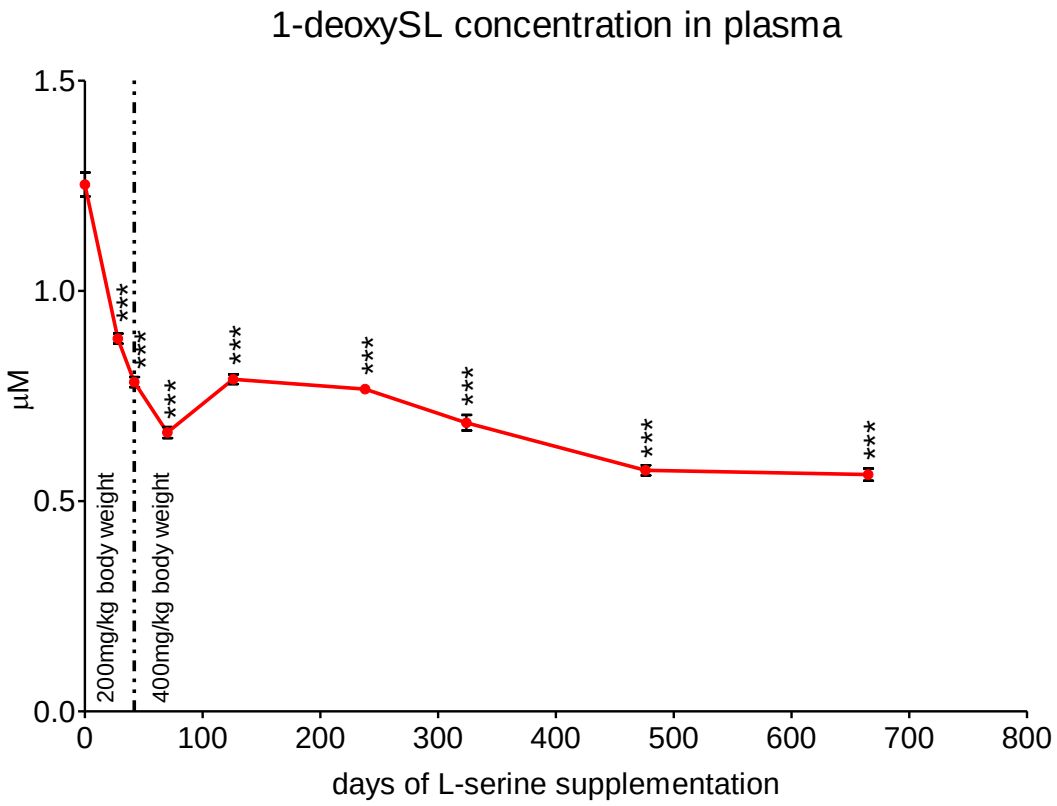


Figure 3-5 Concentration of 1-deoxySLs in plasma of the patient under L-serine supplementation. Baseline concentration of 1-deoxySLs was strongly decreased upon supplementation with 200mg/kg body weight L-serine. After 126 days and with an increased concentration of L-serine (400mg/kg), the decline slowed down and 1-deoxySL concentrations stabilized at ~45% of the values at baseline. All changes of 1-deoxySL concentration were compared to levels at baseline and significance of the difference was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001.

The results of our cell culture model showed that mutations at Ser 331 of SPTLC1 affect not only the activity of SPT with its alternative amino acid substrate L-alanine, but also with the alternative activated fatty acid stearoyl-CoA. The use of stearoyl-CoA by SPT results in the formation of C20-based sphingolipids. Increased concentrations of C20 SLs were exclusively found in cells expressing one of the SPTLC1 p.S331 mutations (p.S331F or p.S331Y) or the SPTLC2 p.I504F mutation, and present a clearly distinctive feature compared to the other nine HSAN1-causing mutations in SPT (see chapter 5 of this thesis).

The measured elevated concentration of C20-based sphingolipids at baseline in our patient (see 3-6) coincided with our observations from cell culture. As for the major C18 SL, also the concentration of the minor C20 SL appeared to be regulated and not affected by the increased L-serine availability. Right after the start of the study, the concentration of C20 SLs dropped significantly and, as observed for the C18-based SLs, returned to the baseline level again, which was reached after 11 months. At the last time point however the concentration of C20 SLs in plasma was again significantly reduced (~25%).

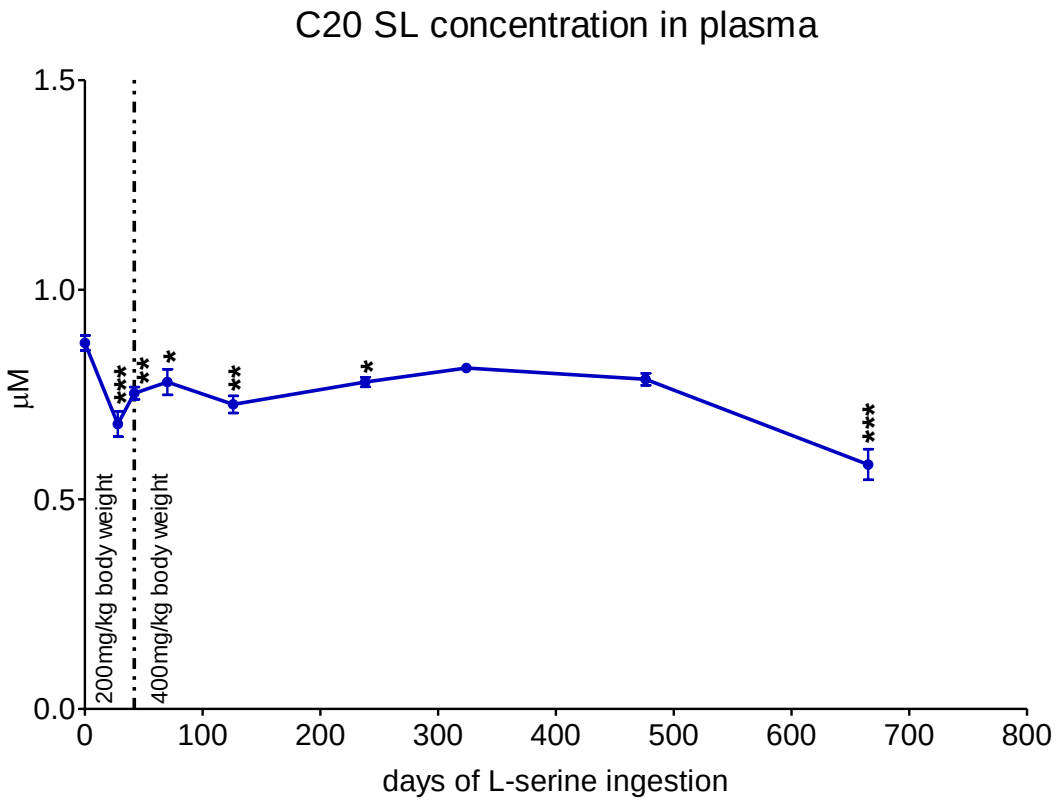


Figure 3-6 Concentration of the minor C20 SLs in plasma of the patient under serine therapy. As seen for 1-deoxySLs, the C20 SLs were severalfolds increased compared to healthy family members. L-serine supplementation did not cause persistent alterations of C20 SL concentrations for 476 days. However the last measurement indicated a decreased C20 SL concentration (~-25%). All changes of C20 SL concentration were compared to levels at baseline and significance of the difference was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001, **p < 0.01, *p < 0.05.

3.5.3 Improvements of the health status under serine therapy:

While on L-serine therapy, the patient and his family noticed and reported positive effects on health status and general constitution of the patient. After 70 days, the hair of the boy had started to grow faster, and the same was observed for his finger and toe nails which started to become thicker and more robust. Wound healing occurred faster and all wounds at the fingers of his left hand completely healed after 70 days of therapy. The patient even reported to suffer less pain while standing.

Accelerated nail and hair growth persisted also after four months. Wound healing further improved, but the skin of the patient was nevertheless very delicate and he still had to wear gloves to prevent the development of new wounds on his fingers. The boy reported increased sweating primarily at the back. He could stand longer without pain and trembling, and passed through the winter season with less upper respiratory tract infections than in the years before. Due to this reduced susceptibility to infections, the use of the cough assist system became necessary only once.

After eight months, the skin of the patient was more robust, so that new blister formations did not occur and even the protective gloves were not necessary anymore. Healing of fresh wounds took 4-5 days, whereas it took 2-3 weeks before the start of the L-serine therapy. The patient felt the temperature of hot water in the bathtub for the first time in his life.

The boy could now stand for about 5 minutes, with a further reduction in trembling.

After 11 months temperature sensation had improved further and the patient reported onset of temperature perception on head, back, thighs, hands, and feet, and the tendency to sweat on the back had further increased. He now felt tingling sensations in his hands and feet. After 16 months of L-serine intake, his endurance in standing without additional support had further improved and it took him longer to fatigue. He had started to feel not only the temperature of hot water, but also temperature differences. Furthermore, the first signs of puberty began.

At the last examination after 22 months of L-serine therapy, the previous improvements in sensitivity, muscular strength and endurance, hair and growth of nails, wound healing, and autonomous sweating had stabilized.

No systematic neuronal examinations of nerve conduction velocities were conducted during the study, so evaluation of the benefit of the L-serine treatment and improvements of the patient are mostly based on subjective reports and observed effects like longer standing times, increased sweating, and improved wound healing.

However, the body weight of the patient was repeatedly measured during several of the follow up examinations and showed constant increase over the whole duration. With a BMI of 11.44 at baseline (see Figure 3-7), the patient was underweight with severe thinness and far below the lowest value of the BMI scale proposed by the world health organization (WHO). During the serine therapy, the mother of the patient repeatedly noticed the highly increased appetite and improved eating behavior of her son. After the intake of L-serine for 22 months, the patient had gained a total 10.2kg or 40% of his initial weight and four BMI points (see Figure 3-7). With 16.04 points, the patient was still underweight but had reached the level of moderate thinness compared to the severe thinness at baseline.

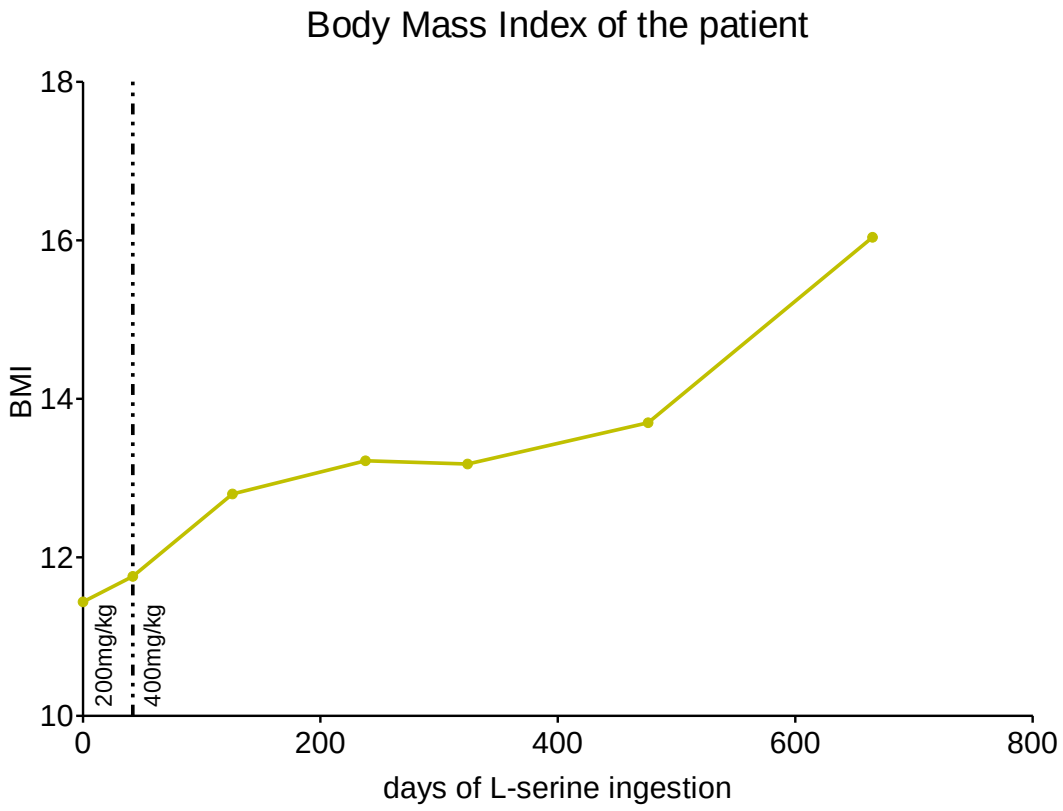


Figure 3-7 BMI of the patient under serine supplementation. Weight of the patient was measured repeatedly over the course of the therapy and BMI was calculated as [kg/m^2].

Figure 3-8 Pictures of patient MGZ-48522 alone in his wheelchair after ~25months of L-serine supplementation and standing without aid next to his six years younger, unaffected brother. Growth difference and hypotrophy is, despite the strong increase in bodyweight, clearly visible. Detailed picture of the hand of the patient shows neither signs of fresh nor poorly healing wounds, nor especially delicate skin at hands and fingertips anymore. Pictures provided by Ekkehard Wilichowski.

3.5.4 Progression of the pathology under serine therapy:

Apart from above mentioned improvements of the patient's health status, negative developments were also observed. After ~6 months of L-serine intake, the patient started to develop a slowly progressing CO_2 retention. After 22 months, blood gas analysis showed a pCO_2 of 60.2mmHg (norm: 35-45mm) at pH 7.375 (norm: 7.35-7.45), with a bicarbonate concentration of 35.2mmol/l (norm: 22-26mmol/l). Symptoms of respiratory insufficiency like dyspnea or tachypnea were not reported by the patient. Other symptoms of hypercapnia were also not found, making the observed degree of hypercapnia even more puzzling. The degree of the CO_2-retention kept aggravating and made nocturnal intermittent self-ventilation inevitable after approximately 2 years.

3.6 Discussion:

The rare inherited peripheral neuropathy HSAN1 presents a remarkable genotypic but also phenotypic variability. Since the first linkage of mutations in SPTLC1 (4, 5) with the disease, nine more mutations in subunits 1 and 2 of SPT have been conclusively associated with HSAN1. However, not only the underlying genotypes but also the caused phenotypes, present with distinguishable characteristics such as the age at onset and the kind and severity of caused symptoms.

Only recently one new mutation of Ser331 (p.S331Y) and one more patient carrying the herein described p.S331F mutation were reported (6, 14). Together with the two already reported HSAN1 patients bearing the p.S331F mutation (9, 13), there are now a total of four independent cases of dominant and non-segregating mutations of SPTLC1 Ser331. All four individual patients present with a severe form of HSAN1 with a characteristic early onset of first symptoms, pronounced muscular hypotrophy, and involvement of the vision and respiratory system (for a summary of the cases and their phenotypes see Table 1).

Mutation/ origin/ Patient No.	S331F / Germany MGZ-48522	S331F / France CMT-791.01	S331Y / Austria	S331F / Korea
Published in:	(13, 15) this study	(9)	(6)	(14)
Age at onset	1,5 - 3	congenital	4	5
Growth retardation	nm	Yes	Yes	nm
Mental retardation	No	Yes	No	nm
Juvenile cataracts	Yes (9)	Yes	Yes (13)	Yes (10)
Vocal cord palsy	No	Yes	No	Yes + hoarsenes
Respiratory problems	Yes	Yes	Yes	Yes
Diffuse muscle hypotrophy, walking aids	Yes Wheelchair (14)	Yes nm	Yes Wheelchair (14)	Yes Walker (27)
Foot ulcers, amputations and/or burns	Yes	Yes	Yes	Yes

Joint hypermobility	Yes	Yes	Yes	Yes
Tremor	nm	nm	Yes	Yes
Fasciculations	nm	nm	Yes	nm
Other ocular manifestations	Yes retinal detachment	nm	No	nm
Additional	Joint contractures	nm	Tremor and scoliosis	Tremor and scoliosis

Table 1 Overview of symptoms and complications observed in patients bearing a mutation of Ser331 in SPTLC1 taken and adapted from (6, 14). Parentheses indicate the age at the event, nm = not mentioned

We compared the biochemical properties of the mutated SPT enzyme *in vitro* (see chapter 5 of this thesis) but also the sphingolipid profiles of patient plasma. We found characteristic patterns which make these mutations an exception amongst all other HSAN1-causing mutations in SPTLC1. In our cell culture model the two mutations of Ser331 not only increased the activity of SPT with its alternative amino acid substrate L-alanine, but they also increased its activity with the canonical substrate L-serine. These mutations even increased the metabolization of the alternative fatty acid substrate stearoyl-CoA. HEK293 cells expressing the p.S331F or p.S331Y variants of SPTLC1 therefore produced significantly higher amounts of the canonical SPT products, the C18-based sphingolipids, the neurotoxic atypical 1-deoxySL, and even the minor C20-based sphingolipids (data not shown here, see chapter 5 of this thesis).

The altered metabolic behavior of the mutated subunit in cell culture was (apart from the increased canonical activity) also present in the patients and could be measured as increased concentrations of the respective sphingolipids in the plasma. The concentrations of 1-deoxySLs, but also C20-based sphingolipids were clearly elevated in three out of four reported patients (MGZ-48522, CMT-791.01 and p.S331Y) (see Figure 3-2). Plasma of the deceased Korean patient (patient no. 4) was not available for analysis. Although 1-deoxySL levels were consistently increased in HSAN1 patients with mutations like p.C133W and p.C133Y (16, 20), this was never the case for the C20-based sphingolipids, making these minor sphingolipid species an additional differentiator for mutations at Ser331.

Supplementation with high doses of L-serine caused increased formation of C18-based sphingolipids. This was seen in cells (see chapter 5), but also to a certain extent in the pilot study with patients bearing the p.C133Y mutation (16). Without stimulation with high doses of L-serine, the p.C133Y mutation did not alter the formation of C18-based sphingolipids in our cell culture model (see chapter 5 of this thesis), nor did any of the tested patients present elevated levels of C18 SLs in their plasma at baseline (see Figure 3-3).

In cells expressing one of the two Ser331 mutations however, we measured a highly significant increase in C18 SL formation even under physiological conditions. This increased canonical activity was not seen for any other mutation of SPTLC1 and represents a unique biochemical property of mutations of Ser331 of SPTLC1.

Increased canonical SPT activity is associated with an increased formation of downstream products of SPT e.g. ceramides and sphingosine-1-phosphate. Elevated levels of ceramides are known to exert pro-apoptotic effects on a large variety of cells and were even discussed as a potential cause for the slow decay of peripheral neurons in HSAN1 (5). Therefore we were relieved to see that the C18 SL levels in our patient, but also in the two other p.S331

patients, did not significantly change under L-serine therapy as compared to the family or other healthy controls (see Figure 3-2 and Figure 3-3). Concentration of C18-based SLs in the plasma of our patient did neither increase during the initial phase of the therapy with low dose L-serine, nor upon the later increase to the established high dose of 400mg/kg bodyweight and day (see Figure 3-4).

This observation conflicts on one hand with our forecast and expectations from cell culture, but on the other hand supports other experiments suggesting the involvement of a rheostat in SPT regulation to maintain a tolerable level of C18-based SLs. This putative rheostat in cell culture induces active degradation of SLs upon increasing *de novo* synthesis, keeping the intracellular SLs at a constant level (data not shown). Obviously this mechanism is not impaired in the p.S331F patient, so that the potential hyperactivity of SPT is compensated for and does not result in increased plasma SL levels.

Within the 22 months of consequent serine therapy, the concentrations of the neurotoxic 1-deoxySLs in plasma of the boy were reduced to 45% of the baseline value, where they apparently reached a plateau and stabilized. Interestingly, the largest decrease occurred within the first 70 days of supplementation. At this first nadir after 70 days, the levels were already lowered by 48%. Of these 70 days, the patient was supplemented with the low dose of L-serine (200mg/kg of body weight and day) for 42 days and took the high dose (400mg) for another 28 days. This implies that even the lower dose of L-serine was already sufficient to competitively inhibit the metabolization of L-alanine by the mutated enzyme. A dose response study would be an appropriate way to determine the optimal concentration range for L-serine, which might be different for the different mutations.

The recovery of 1-deoxySL levels after 70 days however is puzzling and additional experiments and a better understanding of the regulatory mechanisms involved in 1-deoxySL formation will be necessary to explain this phenomenon. The considerable decrease and recovery of the plasma concentration was not only seen for the atypical 1-deoxySLs but also for the canonical C18-based and the minor C20-based sphingolipids. While C18 SLs and C20 SLs follow the same pattern of decrease and recovery, the nadir of this pattern is delayed for the 1-deoxySLs and the full recovery is effectively prevented by the competitive inhibition with L-serine. This argues for distinct separate regulatory effects underlying the formation of 1-deoxySLs and C18- or C20-based sphingolipids, respectively.

However the desired effect took place and the 1-deoxySL concentration in the plasma of the patient was lowered and stabilized at a level of about 50% of the baseline value. Nevertheless, these levels are still 5-folds higher than in healthy controls. A decrease of 95% as observed in the p.C133Y pilot study (16) was not reached. A direct comparison of the two

genotypes is not appropriate, as the resulting phenotypes present pronounced differences in terms of onset and severity of the symptoms. Furthermore, their impacts on the biochemical properties of SPT are also very distinct. Considering the highly increased general activity of SPT in cells expressing the p.S331F mutation, and also the mentioned severe pathology, a 50% reduction of 1-deoxySL concentration, without increasing the C18 SL concentration in the plasma of the patient, are promising results.

The increased concentration of C20–based sphingolipids consistently seen in S331 mutant-expressing cells and also in patient plasma samples argues for another shift in substrate preference, causing increased activity of the mutated SPT complexes with stearoyl-CoA instead of the usually preferred substrate palmitoyl-CoA.

As for the normal C18 SLs, no clear and significant changes of the elevated C20 SL concentrations were observed during the L-serine therapy over the 476 days. The concentrations showed the same nadir after 28 days and recovered slowly afterwards until they reached the baseline levels again after eleven months (324 days). The last checkpoint after 665 days however showed a clear decrease of C20 SL concentration of about 25%. This might represent an outlier but more successive measurements are necessary to verify or refute a trend in this development.

Independent investigations on SPT in yeast had revealed regulatory mechanisms for keeping sphingolipid levels constant, which together with our observations might explain the altered sphingolipid profiles in HEK293 cell expressing the p.Ser331 mutations, but also in the plasma of the patients.

Two orthologues of the yeast protein Tsc3 were identified and characterized in the human genome (small subunit SPT a and b; ssSPTa and ssSPTb) (21). Tsc3 is a small but essential protein needed for full activity of the yeast orthologue of SPT. Co-expression of the small subunits together with SPT in yeast or mammalian cells increased SPT activity 50-100 fold. Besides the induction of SPT activity, the ssSPTs affect the acyl-CoA preference of the enzyme. Co-expression of the human SPT subunits SPTLC1 together with SPTLC2 and ssSPTa in a yeast SPT knockout strain results in the formation of C18-based sphingoid bases, while cells expressing SPTLC1, SPTLC2 and ssSPTb preferred stearoyl-CoA and formed increased amounts of the minor C20-based sphingolipids (21).

Harmon recently showed that yeast SPT knockout cells expressing the mutated human SPTLC1 p.S331F together with the wild type SPTLC2 subunit grow without further activation at permissive temperature, while cells expressing the wild type SPTLC1 needed co-expression of ssSPTa to reach full activity and rescue the knockout phenotype at 26°C. Co-expression of SPTLC1 p. S331F and SPTLC2 together with ssSPTb but not ssSPTa resulted

in an additional increase in SPT activity, allowing growth even at the restrictive temperature of 37°C (22).

Harmon identified a single residue (ssSPTa-M25) which is essential for the proper interaction of ssSPTa and SPTLC1. This interaction is disturbed in the mutant p.S331F by steric inhibition, causing reduced additional activation of SPT due to impaired interaction of both proteins. In ssSPTb, methionine 25 is replaced by valine which does not cause a steric clash with the mutated SPTLC1 p.S331F, and therefore still allows proper interaction and activation of the mutated subunit.

The reported independence of the mutated subunit from additional activation by ssSPTs, although done in the yeast model, explains and supports our own observations of increased canonical activity of SPT in cells expressing one of the Ser331 mutations (see chapter 5).

The finding that the mutation at Ser331 is activated by ssSPTb rather than ssSPTa furthermore explains the observed shift in acyl-CoA preference and increased use of stearoyl-CoA in the condensation reaction. The resulting increased concentrations of the minor C20-based sphingolipids were found in mutant-expressing cells but also in plasma of the affected patients.

Here we report the case of a single patient undergoing a serine therapy trial. A more comprehensive long-term study on the effects of L-serine supplementation in HSAN1 patients is currently in progress. Due to the limited patient number, neurological and general improvements were not systematically assessed and this case report is mostly based on the observations and reports from patients and supervising pediatricians.

Typical symptoms of HSAN1 (e.g. poor wound healing, impaired growth of hair and nails, and skin fragility) improved significantly under serine therapy, which was also reported from participants of the first L-serine pilot study (16). These improvements may be due to the enhanced skin innervation, allowing for a better regulation of perfusion and a faster response to damage.

Under supplementation with L-serine, the patient´s appetite increased and he gained 10kg within the 22 months of therapy. Weight gain and increased muscle strength and endurance shows that even the degree of muscular atrophy and muscular coordination improved within the 22 months of the trial. This again might be based on better innervation of the skeletal musculature by motoric nerves or enhanced performance of the remaining myelinated nerve fibers. Although HSAN1 is a primarily axonal neuropathy, the measured motoric NCVs of the patient suggest marked demyelination of motoric nerves as well. Loss of nerve fibers and demyelination are common conditions of HSAN1 and were described in 1951 already (23).

However, demyelination bears higher potential for a fast recovery (24, 25) and therefore might provide an explanation for the improvements in muscular coordination and strength. Muscle and motoric nerve biopsies could have proven or contradicted this hypothesis but were not done, as the assessment of the exact processes behind the improvements were beyond the scope of this study. The pronounced and constant weight gain however raises the hope that global hypotrophy of the patient despite the duration of the disease might keep improving.

The aggravating respiratory problems observed seem to be a distinct feature for mutations of SPTLC1 Ser331, as all four reported patients presented more or less pronounced impairments of their respiratory system. Although the mother of our patient reported decreased susceptibility for infections of the upper respiratory system und reduced necessity for the use of the cough assist system, the patient presented symptoms of CO_2-retention after 6 months of therapy. After 22 months, the patient was diagnosed with metabolic compensated CO_2-retention and was finally subjected to nocturnal intermittent self-ventilation.

Sphingolipids have repeatedly been shown to play a role in several lung disorders (26, 27). These studies mostly focused on the counteracting effects of ceramides and sphingosine-1-phosphate in chronic and inflammatory lung disease. However, nothing is known about the effects of normal C18-based sphingolipids and especially the atypical 1-deoxySLs on CO_2 retention or hypercapnia. This case report is the first report describing these complications in the context of a severe form of HSAN1. Signs of respiratory insufficiency like dyspnea or tachypnea were not reported, and symptoms of hypercapnia were also absent. Improvements in infection resistance of the upper respiratory tract and absence of symptoms of mechanical impairments and insufficiency of ventilation however argue for an unknown metabolic cause for the observed CO_2 retention rather than purely muscular atrophy and poor ventilation.

3.7 References for chapter 3:

1. Rotthier, A., Baets, J., Timmerman, V., and Janssens, K. (2012) Mechanisms of disease in hereditary sensory and autonomic neuropathies. *Nat. Rev. Neurol.* **8**, 73–85

2. Dyck, P. J. (1993) Neuronal atrophy and degeneration predominantly affecting peripheral sensory and autonomic neurons. in *Peripheral Neuropathy*, 3rd Editio (Dyck, P. J., Thomas, K. P., Griffin, J. W., Low, P. A., and Poduslo, J. F. eds), pp. 1065–1093, Saunders Philladelphia

3. Houlden, H., King, R., Blake, J., Groves, M., Love, S., Woodward, C., Hammans, S., Nicoll, J., Lennox, G., O'Donovan, D. G., Gabriel, C., Thomas, P. K., and Reilly, M. M. (2006) Clinical, pathological and genetic characterization of hereditary sensory and autonomic neuropathy type 1 (HSAN I). *Brain*. **129**, 411–25

4. Bejaoui, K., Wu, C., Scheffler, M. D., Haan, G., Ashby, P., Wu, L., de Jong, P., and Brown, R. H. (2001) SPTLC1 is mutated in hereditary sensory neuropathy, type 1. *Nat. Genet.* **27**, 261–2

5. Dawkins, J. L., Hulme, D. J., Brahmbhatt, S. B., Auer-Grumbach, M., and Nicholson, G. a (2001) Mutations in SPTLC1, encoding serine palmitoyltransferase, long chain base subunit-1, cause hereditary sensory neuropathy type I. *Nat. Genet.* **27**, 309–12

6. Auer-Grumbach, M., Bode, H., Pieber, T. R., Schabhüttl, M., Fischer, D., Seidl, R., Graf, E., Wieland, T., Schuh, R., Vacariu, G., Grill, F., Timmerman, V., Strom, T. M., and Hornemann, T. (2013) Mutations at Ser331 in the HSN type I gene SPTLC1 are associated with a distinct syndromic phenotype. *Eur. J. Med. Genet.* **56**, 266–9

7. Murphy, S. M., Ernst, D., Wei, Y., Laurà, M., Liu, Y.-T., Polke, J., Blake, J., Winer, J., Houlden, H., Hornemann, T., and Reilly, M. M. (2013) Hereditary sensory and autonomic neuropathy type 1 (HSANI) caused by a novel mutation in SPTLC2. *Neurology*. **80**, 2106–11

8. Rautenstrauss, B., Neitzel, B., Muench, C., Haas, J., and Holinski-Feder, E. (2009) LATE ONSET HEREDITARY SENSORY NEUROPATHY TYPE 1 (HSN1) CAUSED BY A NOVEL P.C133R MISSENSE MUTATION IN SPTLC1 Würzburg, Germany. in *2009 Meeting of the Peripheral Nerve Society July 4-8, 2009*, p. 290 of 381

9. Rotthier, A., Baets, J., De Vriendt, E., Jacobs, A., Auer-Grumbach, M., Lévy, N., Bonello-Palot, N., Kilic, S. S., Weis, J., Nascimento, A., Swinkels, M., Kruyt, M. C., Jordanova, A., De Jonghe, P., and Timmerman, V. (2009) Genes for hereditary sensory and autonomic neuropathies: a genotype-phenotype correlation. *Brain*. **132**, 2699–711

10. Rotthier, A., Auer-Grumbach, M., Janssens, K., Baets, J., Penno, A., Almeida-Souza, L., Van Hoof, K., Jacobs, A., De Vriendt, E., Schlotter-Weigel, B., Löscher, W., Vondráček, P., Seeman, P., De Jonghe, P., Van Dijck, P., Jordanova, A., Hornemann, T., and Timmerman, V. (2010) Mutations in the SPTLC2 subunit of serine palmitoyltransferase cause hereditary sensory and autonomic neuropathy type I. *Am. J. Hum. Genet.* **87**, 513–22

11. Ernst, D., Murphy, S. M., Sathiyanadan, K., Wei, Y., Othman, A., Laura, M., Donaghy, M., Houlden, H., Reilly, M. M., and Hornemann, T. (2015) Novel HSAN1

Mutation in Serine Palmitoyltransferase Resides at a Putative Phosphorylation Site That Is Involved in Regulating Substrate Specificity. *NeuroMolecular Med.* **17**, 47–57

12. Penno, A., Reilly, M. M., Houlden, H., Laurá, M., Rentsch, K., Niederkofler, V., Stoeckli, E. T., Nicholson, G., Eichler, F., Brown, R. H., von Eckardstein, A., and Hornemann, T. (2010) Hereditary sensory neuropathy type 1 is caused by the accumulation of two neurotoxic sphingolipids. *J. Biol. Chem.* **285**, 11178–87

13. Rotthier, A., Penno, A., Rautenstrauss, B., Auer-Grumbach, M., Stettner, G. M., Asselbergh, B., Van Hoof, K., Sticht, H., Lévy, N., Timmerman, V., Hornemann, T., and Janssens, K. (2011) Characterization of two mutations in the SPTLC1 subunit of serine palmitoyltransferase associated with hereditary sensory and autonomic neuropathy type I. *Hum. Mutat.* **32**, E2211–25

14. Suh, B. C., Hong, Y. Bin, Nakhro, K., Nam, S. H., Chung, K. W., and Choi, B.-O. (2014) Early-onset severe hereditary sensory and autonomic neuropathy type 1 with S331F SPTLC1 mutation. *Mol. Med. Rep.* **9**, 481–6

15. Huehne, K., Zweier, C., Raab, K., Odent, S., Bonnaure-Mallet, M., Sixou, J.-L., Landrieu, P., Goizet, C., Sarlangue, J., Baumann, M., Eggermann, T., Rauch, A., Ruppert, S., Stettner, G. M., and Rautenstrauss, B. (2008) Novel missense, insertion and deletion mutations in the neurotrophic tyrosine kinase receptor type 1 gene (NTRK1) associated with congenital insensitivity to pain with anhidrosis. *Neuromuscul. Disord.* **18**, 159–66

16. Garofalo, K., Penno, A., and Schmidt, B. (2011) Oral l-serine supplementation reduces production of neurotoxic deoxysphingolipids in mice and humans with hereditary sensory autonomic neuropathy type 1. *J. Clin. …*. 10.1172/JCI57549.)

17. Swanson, A. G. (1963) Congenital Insensitivity to Pain with Anhydrosis. *Arch. Neurol.* **8**, 299

18. Mullen, T. D., Hannun, Y. A., and Obeid, L. M. (2012) Ceramide synthases at the centre of sphingolipid metabolism and biology. *Biochem. J.* **441**, 789–802

19. Ogretmen, B., and Hannun, Y. A. (2004) Biologically active sphingolipids in cancer pathogenesis and treatment. *Nat. Rev. Cancer*. **4**, 604–16

20. Laurá, M., Murphy, S. M., Hornemann, T., Bode, H., Polke, J., Blake, J., Houlden, H., and Reilly, M. M. (2012) P42 Hereditary sensory neuropathy type 1: correlation of severity and plasma atypical deoxy-sphyngoid bases. *Neuromuscul. Disord.* **22**, S18

21. Han, G., Gupta, S. D., Gable, K., Niranjanakumari, S., Moitra, P., Eichler, F., Brown, R. H., Harmon, J. M., and Dunn, T. M. (2009) Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 8186–91

22. Harmon, J. M., Bacikova, D., Gable, K., Gupta, S. D., Han, G., Sengupta, N., Somashekarappa, N., and Dunn, T. M. (2013) Topological and functional characterization of the ssSPTs, small activating subunits of serine palmitoyltransferase. *J. Biol. Chem.* **288**, 10144–53

23. Denny-Brown, D. (1951) Hereditary sensory radicular neuropathy. *J. Neurol. Neurosurg. Psychiatry*. [online] http://jnnp.bmj.com/content/14/4/237 (Accessed September 3, 2012)

24. Thomas, P. K., and Ochoa, J. (1993) Clinical features and differential diagnosis. in *Peripheral Neuropathy*, 3rd Editio (Dyck, P. J., Thomas, K. P., Griffin, J. W., Low, P. A., and Poduslo, J. F. eds), pp. 749–774, Saunders Philladelphia

25. Thomas, P. K. (1970) Peripheral neuropathy. *Br. Med. J.* **1**, 349–351

26. Tibboel, J., Reiss, I., de Jongste, J. C., and Post, M. (2014) Sphingolipids in lung growth and repair. *Chest*. **145**, 120–8

27. Yang, Y., and Uhlig, S. (2011) The role of sphingolipids in respiratory disease. *Ther. Adv. Respir. Dis.* **5**, 325–44

4 Hereditary sensory and autonomic neuropathy type 1 in a German family is not caused by the new mutation p.A339V in SPTLC1 alone.

Heiko Bode, Kristl Claeys, Joachim Weis, Björn Tackenberg, Bernd Rautenstrauss, Thorsten Hornemann

Unpublished manuscript

This Chapter has not been published in a peer reviewed journal. The clinical description of the patient and the genotypes of the family were provided by the neurologists in charge, Kristl Claeys and Joachim Weis. Plasma of the index patient and his family was kindly provided by Kristl Claeys.

I transfected this new mutation into HEK293 cells and generated the stable mutant-expressing cell line. I measured *de novo* production of canonical and atypical sphingolipids by those cells in various conditions and also the concentrations of sphingolipids and especially 1-deoxySA and 1-deoxySO in the plasma of the patient his family. These data are presented in Figure 4-2 – Figure 4-8.

4.1 Abstract:

The ubiquitous enzyme serine palmitoyltransferase (SPT) catalyzes the first and rate-limiting step of sphingolipid *de novo* synthesis. The canonical reaction is the condensation of the activated fatty acid palmitoyl-CoA and the amino acid L-serine, giving rise to the first intermediate sphingoid base 3-keto-sphinganine. Mutations in SPT cause the rare neurological disorder, “hereditary autonomic and sensory neuropathy type 1” (HSAN1). Several mutations in the serine palmitoyltransferase long chain base subunits 1 and 2 (SPTLC1 and 2) impair the substrate specificity of the formed SPT complex and cause an increased activity with L-alanine and glycine respectively. The use of these alternative amino acids in the condensation reaction results in the formation of the atypical and neurotoxic 1-deoxysphingolipids. Elevated concentrations of these atypical sphingolipids are consistently measured in HSAN1 patient plasma and represent a crucial hallmark and biomarker for the disease. Increased activity of mutated SPT with L-alanine was observed upon overexpression of the mutations in HEK293 cells but also in lymphoblasts from HSAN1 patients.

Here we report a new mutation causing an exchange of Alanine339 for Valine (p.A339V) in the sequence of SPTLC1, which was found in a German family suffering from an autosomal dominant inherited peripheral neuropathy. The index case was diagnosed with HSAN1 and the heterozygous mutation was identified upon sequencing known candidate genes including, SPTLC1 and SPTLC2. Further analysis of the family revealed three more clinically affected individuals, but only two out these three patients carried the p.A339V mutation. All clinically affected members had elevated concentrations of 1-deoxySLs in their plasma.

In vitro investigation of the biochemical properties of the p.A339V mutation revealed only mild effects on substrate specificity and canonical activity of SPT. The mutation was absent in two of the four clinically affected male members of the family, while it was present in the clinically and biochemically unaffected daughter of the index case. Therefore the SPTLC1 variant p.A339V cannot be the only cause of the phenotype, and we conclude that the it does not cause HSAN1 in this family. Rather another, yet unknown metabolic or regulatory process must be involved in the increased formation of 1-deoxySLs in this family, thereby causing the neuropathy.

4.2 Introduction:

Seven subtypes of hereditary sensory and autonomic neuropathies (HSAN1-7), which are caused by mutations in at least 14 different genes, are annotated in the OMIM database (1–3). All of these genotypes cause phenotypes with characteristic symptoms such as the loss of sensation of pain and temperature in distal limbs, frequently leading to the formation of chronic ulcerations on feet and hands which can even progress to osteomyelitis and the necessity of amputations.

The autosomal dominant inherited subtype HSAN1 is caused by mutations in five different genes: SPTLC1, SPTLC2, ATL, DNMT1, and RAB7 (1). Mutations in SPTLC1 are the most frequent cause of HSAN1 (15). Seven different mutations in serine palmitoyltransferase long chain base subunit 1 (SPTLC1) and another six mutations in SPTLC2 are known to cause the HSAN1 phenotype with variable severity (6–14).

HSAN1 is characterized by a late onset between the 2nd and 5th decade, a slow progressive sensory loss, and motor nerve degeneration. Plantar ulcers develop predominantly at the lower limbs and frequently develop into chronic complications and necessity of amputation (4). Lancinating pain attacks in the extremities are typically present only in the SPT-associated forms of HSAN1 (5).

The canonical function of the affected enzyme serine palmitoyltransferase (SPT) is the catalysis of the first and rate limiting step of sphingolipid (SL) *de novo* synthesis, the condensation of the activated fatty acid palmitoyl-CoA and the amino acid L-serine (16). Several of the above mentioned mutations in SPT have been shown to cause an increased substrate promiscuity *in vitro*, resulting in higher activity of SPT with its alternative substrates L-alanine and glycine. Condensation of palmitoyl-CoA with L-alanine results in the formation of 1-deoxysphinganine (1-deoxySA), while the use of glycine generates 1-desoxymethylsphinganine (1-desoxyMeSA). These two compounds build the base for an atypical class of 1-deoxysphingolipids (1-deoxySLs) which was shown to be neurotoxic (17).

Elevated levels of 1-deoxySLs can be measured consistently in the plasma of all HSAN1 patients with mutations in SPT (9, 11, 17, 18) and correlate with the severity of presented symptoms (19). Therefore, they represent a unique hallmark and reliable biomarker for the disease.

The specific phenotypes and severity of the symptoms caused by the different mutations in SPT are heterogeneous, which is reflected by the genotype-specific average age at onset, ranging from congenital up to 70 years (12, 20), but also in the general speed of progression

of the disease and presence of additional symptoms such as ocular manifestations and growth retardation (6, 11, 21).

Here we report a new mutation of SPTLC1 which was found in a German family suffering from dominantly inherited sensory and autonomic neuropathy. The index case (born 1937) presented with typical symptoms of HSAN1 affecting predominantly his lower limbs. He showed delicate skin and frequent formation of blisters, poor wound healing, and episodes of spontaneous shooting pains in the limbs. Ulcerations and osteomyelitis had not developed, possibly because they were prevented by early interference with medical foot care and orthopedic shoes. The late onset of the disease furthermore suggested a rather mild phenotype. Two sons and the brother of the primary case presented with symptoms of peripheral neuropathy as well, but the severity of the symptoms was quite variable within the family.

The initial diagnosis of the index case was HSAN1 and was further confirmed by the identification of the p.A339V mutation in SPTLC1. However, this variant was absent in the brother and oldest son of the index case.

We measured the plasma sphingolipd profiles of five members of this family. To investigate the biochemical characteristics of the new mutation, we overexpressed the mutant in HEK293 cells. We included these cells in the biochemical comparison of all SPT mutations (see chapter 5 of this thesis).

4.3 The patients:

The index case II:1 (see Figure 4-1) is a German male born on 21.12.1937. He suffers from peripheral neuropathy predominantly affecting the lower limbs. Delicate skin at the feet, frequent formation of blisters, along with poor wound healing at the feet and shooting neuropathic pains in the lower limbs are the major symptoms presented by this patient. Furthermore, he reported slowly progressing numbness and reduced sensation of temperature and pressure spreading from the feet into the calves. Fine motor skills had started to decline and sporadic shooting pains had emerged in the left forearm. Normal sweating upon physical activity started in a delayed manner and with lower intensity.

Analysis of the whole family revealed three more male members which showed first neurological abnormalities or were already diagnosed with peripheral polyneuropathy like the index case (see Figure 4-1). The younger brother II:5 (*1940) as well as the youngest son III:5 (*Dec. 1966) reported strong impairments by spontaneous neuropathic pain and poor wound healing, as observed in the index case. The first-born son III:1 (*1964) did not suffer from neuropathy yet but presented first abnormalities during neurologic and electroneurographical examination. Neither the Sister II:3 (*1939) nor the daughter III:3 (*Jan1966) showed signs of neurological impairments.

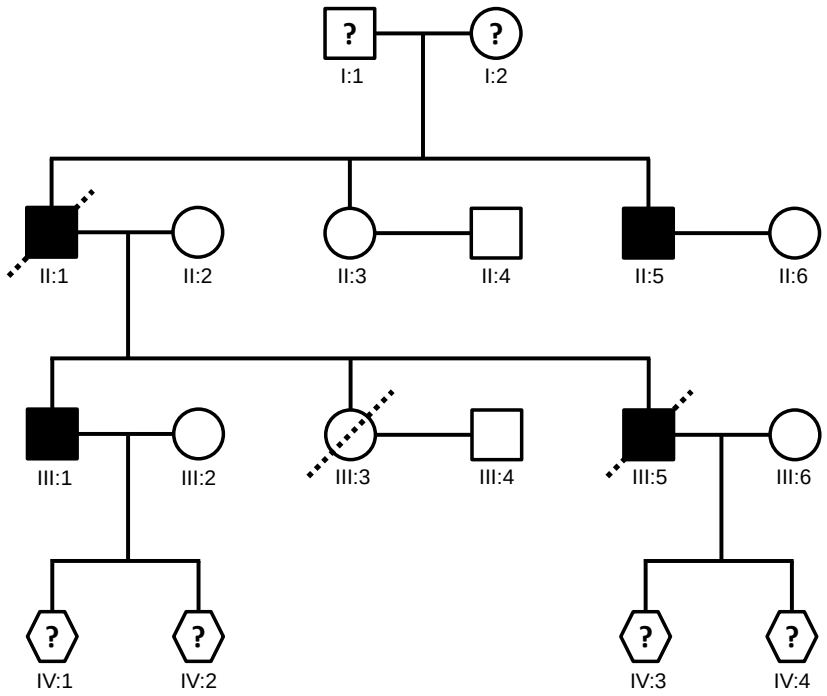


Figure 4-1 Pedigree of the index case (II:1) carrying the p.A339V variant of SPTLC1. Boxes represent male individuals, circles are females. Filled symbols indicate the presence of neurological symptoms or peripheral neuropathy with variable severity. Dashed diagonal lines mark the identified carriers of the p.A339V mutation. No genomic or clinical data is available from the deceased parents (I:1-2) and healthy grandchildren (IV:1-4) of the index case, as indicated by the question marks.

Genomic sequencing of HSAN1-associated genes was conducted to investigate the underlying cause of the neuropathy in the index case II:1 and the other affected male members of the family. A yet unknown heterozygous missense mutation in SPTLC1 (C-to-T replacement at nucleotide 1016 causing an Alanine to Valine exchange at residue 339 → p.A339V) was identified in the index case and concordantly also in the genomes of his daughter III:3 and his youngest son III:5. Further mutations in other HSAN1-causing genes like SPTLC1, 2, 3, Rab7, and Atlastin1 were not found in the index case II:1, his daughter III:3,his son III:5, the sister II:3, the brother II:5 nor the first son III:1.

4.4 Methods:

4.4.1 Cloning and expression of the mutation:

To investigate the effects of the p.A339V exchange on the function of the SPT holoenzyme, we cloned the mutation into our mammalian expression vector pcDNA™3.1/V5-His TOPO® (Invitrogen) (22), using site directed mutagenesis of the SPTLC1 vector with the mutation primers:

SPTLC1_p.A339V_for 5'-gggatacTGCTTTTCAGtaagcTTACCTcccctgttagc-3'
SPTLC1_p.A339V_rev 5'-gctaacaggggAGGTAAgcttaCTGAAAAGCAgtatccc-3'

Expression constructs for the wild type control and p.C133W mutation were generated using the following sequences:

SPTLC1_for 5'-caccatggcgaccgccacggagcagtgggttc-3'
SPTLC1_rev 5'-gagca- ggacggcctgggctacctcc-3'

SPTLC1_p.C133W_for 5′-ggggacccagaggattttatggcacattt gatgttc-3′
SPTLC1_p.C133W_rev 5′-aggtacccacgcca tacttctttagagatgctaaagc-3′

The primers for the p.A339V construct were modified to create an additional silent HindIII (aagcTT) restriction site right after the mutation site.

Correct insertion of the nucleotide exchange was confirmed by sequencing of the amplified transfection vectors with an Applied Biosystems 310 Genetic Analyzer, using the BigDye Terminator v3.0 cycle sequencing kit.

Mutation-containing plasmids were cloned and finally transfected into HEK293 cells (ATCC) using Lipofectamine 2000 (Invitrogen). Cells expressing the mutated SPTLC1 were selected by permanent supplementation of the standard growth medium (DMEM supplemented with 1xPenicillin/Streptomycin mix (both from SIGMA) and 10% fetal bovine serum (Fisher scientific FSA15-043)) with 400µg/ml G418 (Gibco).

Expression of the mutated subunits in the HEK293 background was confirmed by western blotting, using antibodies against the V5 and His tag of the expression plasmid. GAPDH was used as loading control.

4.4.2 Cell culture-based SPT activity assay:

Two hundred and fifty thousand cells per well were seeded in 2ml fresh medium in 6-well plates (BD Falcon) and pre incubated for two more days until ~80-90% confluency. this preincubation, the selection medium was exchanged for L-serine and L-alanine-free DMEM (Genaxxon Bioscience, Ulm, Germany), containing 10% FBS and 1% P/S and 400µg/ml G418.

For the d3 metabolic labeling assay, we added 2mM (10mM for the high dose approach) of the deuterium-labeled substrate (2,3,3,3)-d4-L-alanine and 1mM of d3-N15-L-serine two hours after the medium exchange described above. A further 24 hours later, cells were harvested in 1ml of cold PBS, counted (Beckman Coulter Z2), pelleted at 800rcf and 4°C, and finally stored frozen at -20°C until further processing.

4.4.3 LC-MS measurement of SL profiles from cells and patient plasma:

Cell pellets were resuspended in 100µl phosphate buffered saline (PBS), respectively 100µl of plasma were aliquoted into a fresh 2ml microreaction tube. Subsequently 500µl MeOH (Honeywell), including the deuterated internal standards d7-sphinganine (d7-SA) and d7-sphingosine (d7-SO) 200pmol each (Avanti Polar lipds, Alabaster CA) were added, and lipids were extracted for 1h under constant agitation with 1400rpm at 37°C on a Eppendorf Thermomixer Comfort (Eppendorf, Hamburg, Germany). Proteins were removed by centrifugation for 5min at 16100rcf and 22°C in an Eppendorf 5415 table top centrifuge (Eppendorf, Hamburg, Germany), 500µl of the supernatant were transferred to fresh tubes, and 75µl of HCl (32%, Sigma) were added. The extracted lipids were hydrolyzed for at least 12 hours at 65°C. Acid hydrolysis was stopped the next day by addition of 100µl KOH [10M], followed by addition of 125µl CHCl3, then another 500µl CHCl3, 100µl NH4OH [2N], and finally 500µl alkaline H2O. Samples were mixed by vortexing after every addition. Phase separation was achieved by centrifugation at 16100rcf and 22°C for 5min. The upper polar phase was removed, using a Pasteur pipette and vacuum pump (BVC21 NT Vario, Vacuubrand, Theilingen, Schweiz). The chloroform phase was washed two more times with 1ml alkaline H2O. The remaining CHCl3 phase was evaporated under constant N2-flow in a Techne Sample Concentrator (Bibby Scientific Ltd, Staffordshire, UK) for ~20min. Dried lipids were stored at -20°C.

4.4.4 Derivatization and LC-MS:

Dried lipids were redissolved in 75µl of derivatization mix (56.7% MeOH, 33.3% EtOH, 10% H2O) and derivatized with 5µl of OPA working solution (990µl boric acid [3%] + 10µl o-Phthalaldehyde [50mg/ml in EtOH] + 0.5µl 2-Mercaptoethanol).

Samples were separated on a reverse phase C18 column (Uptispere 120 Å, 5µm, 125x2 mm, Interchim, France) at a flowrate of 400µl/min. Mass spectra were recorded in full scan positive ion mode by a triple quad detector (TSQ quantum ultra, Thermo scientific) and were verified and quantified using Xcalibur (Thermo). Statistical analysis was conducted using GraphPad Prism 5.

4.6 Results:

4.6.1 Typical and atypical sphingolipid profiles in plasma of the index case and his family:

We measured the SL profiles of plasma samples from the index case (m II:1), his siblings (sister II:3 and brother II:5) and his consanguineous offspring (two males and one female III:1, III:3 and III:5). Significantly elevated concentrations of C18-sphingosine and sphinganine were observed in the brother (m II:5), son no. 1 (m III:1) and with minor significance also in the daughter (f III:3) of the index case (see Figure 4-2).

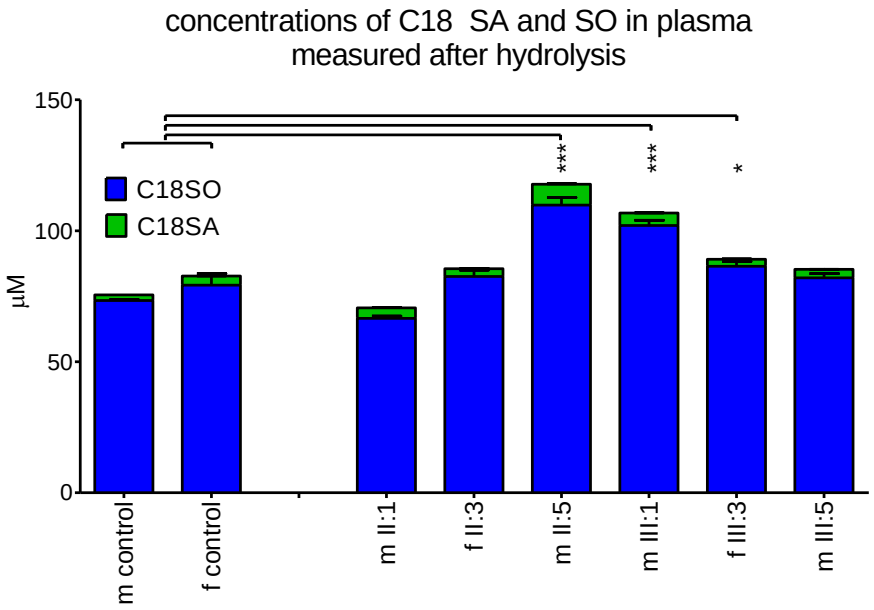


Figure 4-2 Concentrations of C18 sphingolipids in plasma samples of the male index case (m II:1), his siblings and children in comparison to samples from healthy, unrelated control individuals. Significance of differences was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001, **p < 0.01 and *p < 0.05.

Apart from the canonical C18 sphingolipids, we also analyzed several other species of sphingolipids. The concentrations of minor C20-based sphingolipids followed the same pattern as seen for the canonical C18 sphingolipids. Brother II:5 and son III:3 showed significantly increased concentrations of these minor sphingolipid species (see Figure 4-3).

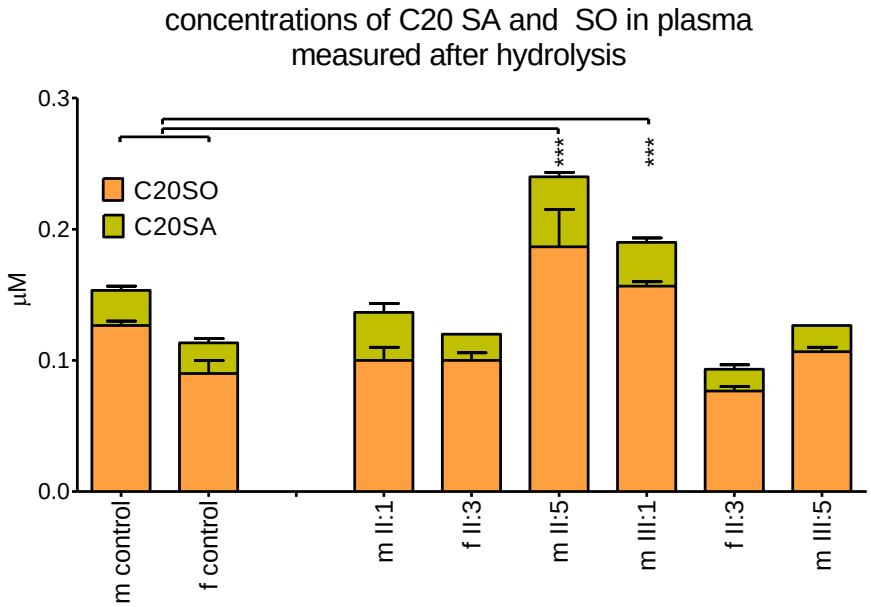


Figure 4-3 Concentration of C20 SLs in plasma of the family and healthy controls. Increased concentrations with p<0.01 were found only in the brother and son no. 1 of the index case, and were absent in all other samples. Significance of differences was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001.

We were especially interested in determining the concentrations of the atypical 1-deoxySLs in this family. The concentrations of 1-deoxySLs were elevated in all family members except for the unaffected sister f II:3 (see Figure 4-4). The brother (m I.*) and son no.1 (m III:1) of the index case again showed the highest concentrations, while the daughter (f III:3) had intermediate levels which were nevertheless significantly increased in comparison to the non-related healthy controls (see Figure 4-4).

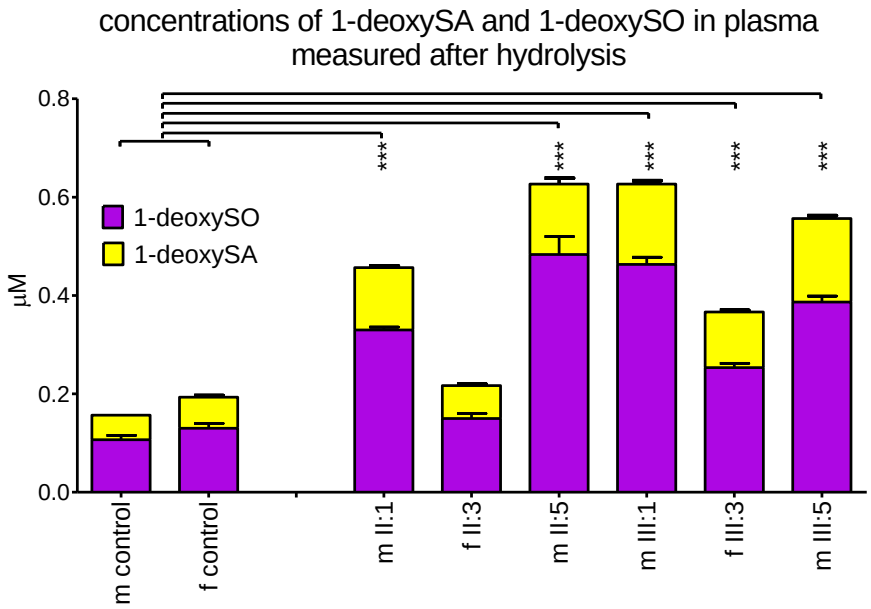


Figure 4-4: 1-Deoxysphingolipid concentration in plasma of the index case (m II:1) and his family in comparison to healthy controls (male and female control). Significance of differences was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001.

4.6.2 Biochemical investigations of the A339V mutation:

We used HEK293 cells as a model to transfect and express the mutated SPTLC1 subunits in a human background. We measured the *de novo* production of normal C18-based sphingolipids as a marker for the functionality of the intracellularly formed SPT complexes. As controls, we included the corresponding wild type subunit SPTLC1 and the HSAN1-causing mutant p.C133W in the assays. For the canonical function without further stimulation or inhibition of SPT activity, we did not find any significant changes in the *de novo* activity of SPT between the A339V mutant, the C133W mutant, the SPTLC1 expressing cells (see Figure 4-5).

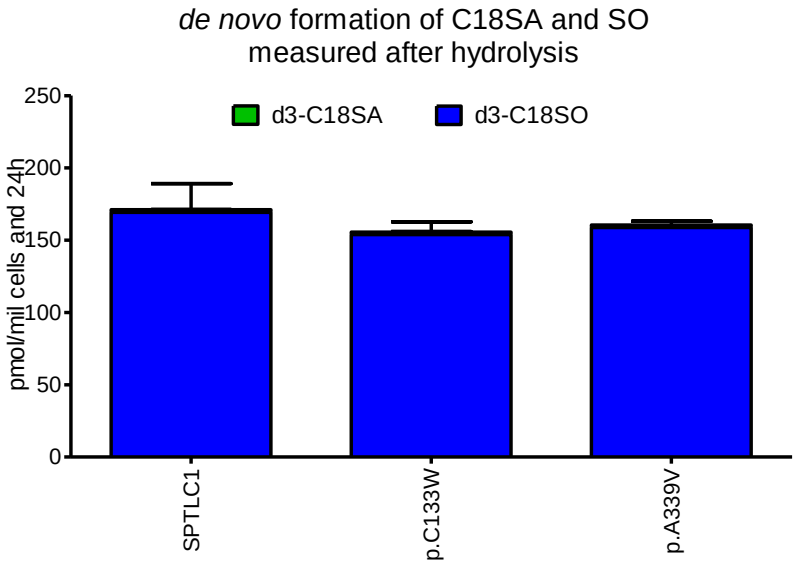


Figure 4-5 Canonical activity of SPT in HEK293 cells overexpressing the wild type SPTLC1, or the SPTLC1 variants p.C133W or p.A339V, respectively. Activity was measured as *de novo* formation of deuterium labelled (d3) C18 SLs arising from d3-N15-L-serine within 24h. Significance of the differences was assessed by 1-way ANOVA followed by Dunnett's post test. Differences were not significant.

The activity of SPT with its alternative substrate L-alanine was increased in cells expressing the p.C133W mutation, while the cells expressing the new p.A339V mutation showed no abnormal activity in comparison to the SPTLC1 wild type (see Figure 4-6).

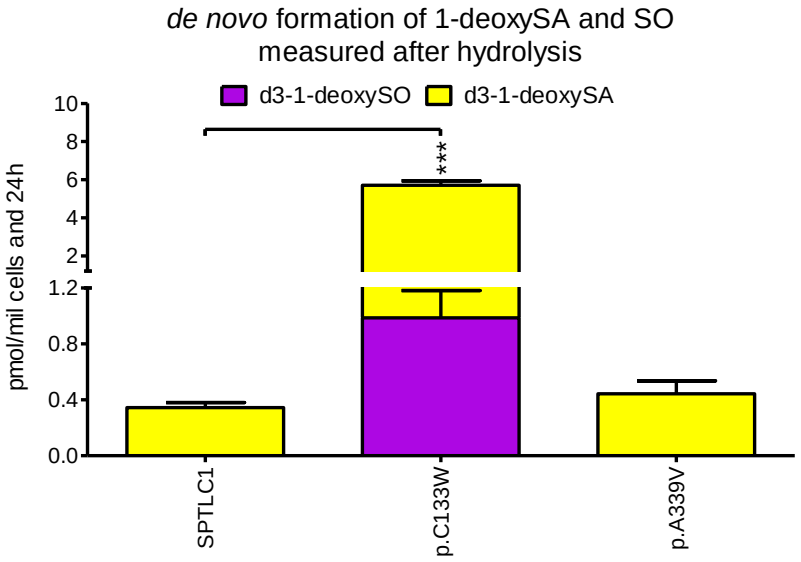


Figure 4-6 *De novo* formation of 1-deoxySLs in the three cell lines. Cells expressing the HSAN1-mutation p.C133W showed >10-folds increased activity with alanine. Expression of p.A339V did not affect 1-deoxySL production. Significance was determined by 1-way ANOVA followed by Dunnett's multiple comparison test, with * signifying p<0.001.**

Until now only 8 out of the 17 mutations in the subunits of SPT (SPTLC1 and SPTLC2) have been shown to increase SPT activity with L-alanine, but increased 1-deoxySL concentrations are consistently found in the plasma of all affected patients and represent a hallmark of the disease. Therefore, we decided to further challenge the cells by treating them with 10mM L-alanine + 1mM L-serine and again measured the *de novo* formation of normal C18 SLs and the atypical 1-deoxySLs. In the presence of high alanine levels we observed a lower activity of SPT with the canonical substrate L-serine in both mutants. The decrease in canonical activity was significant ($p<0.001$) for cells expressing the p.C133W mutation and for the p.A339V cells ($p<0.05$) (see Figure 4-7).

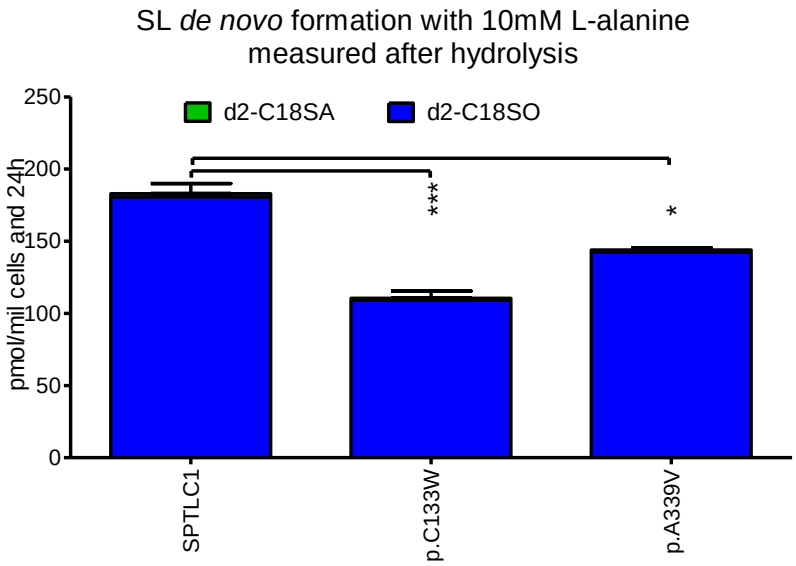


Figure 4-7 Canonical activity of SPT with excess availability of its alternative substrate L-alanine. Under these conditions, expression of the p.C133W and p.A339V mutations significantly reduced SPT activity with the canonical substrate L-serine. Significance was determined by 1-way ANOVA followed by Dunnett's multiple comparison test with ***p<0.001 and *p<0.05.

The formation of 1-deoxySLs was altered by the increased alanine concentration in the medium as well. Expression of the mutated subunits caused a several fold higher activity with L-alanine, resulting in elevated 1-deoxySL concentrations. As before, this increased activity was highly significant for the cells expressing the p.C133W variant and less pronounced but still significant at the p<0.05 level for the cells expressing the p.A339V mutation (see Figure 4-8).

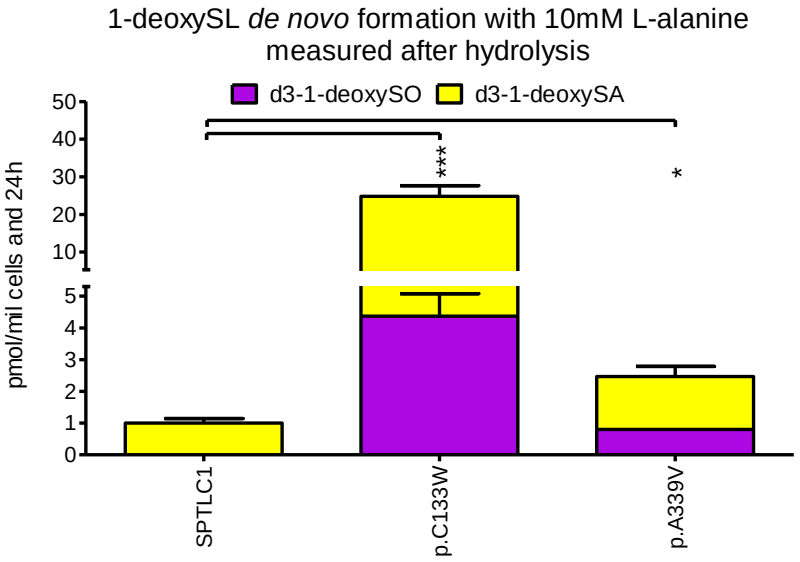


Figure 4-8 *De novo* formation of 1-deoxySLs in p.C133W and p.A339V cells with supplementation of 10mM L-alanine. Significance of differences was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001 and *p < 0.05.

4.7 Discussion:

The pedigree of the family presented here indicates a strong male to male transmission of the phenotype which does not correlate with the segregation of the SPTLC1 p.A339V genotype. The index case II:1 and his youngest son III:5 carry the mutation and present with the classical HSAN1 symptoms e.g. formation of slowly healing blisters, impaired pain and temperature sensation, and chronic neuropathic pains. The brother II:5 shows similar symptoms and severity as the index case II:1 and his son III:5. However, neither p.A339V, nor any other typically HSAN1-associated mutation were detected in the genome of II:5. The mutation was also absent in the older son III:1, who presented only minimal abnormalities during neurologic and electroneurographic examinations and did not suffer from any neuropathy-related impairments until today. The sister II:3 of the index patient tested negative for the mutation. She was not further examined in detail as she did not show or report any sign of a peripheral neuropathy, as seen in her brothers and nephews. The daughter III:3 had no signs of neuropathy, which was confirmed during thorough neurological examinations, but the genetic analysis revealed that she carries the heterozygous p.A339V mutation as well.

The inconsistent segregation of the p.A339V mutation with the clinical phenotype questions its causative role in the neuropathy and makes it an unlikely candidate, at least as the sole cause for the neurological complications in this family. Two family members which are affected by the disease (the son only very mildly) were tested negative for the mutation. On the other hand the daughter, who does not present with any symptoms, carries the mutation. HSAN1 caused by mutations in SPT is an autosomal dominant trait. The absence of neurological symptoms in the daughter therefore clearly contradicts the causative role of the p.A339V mutation. Furthermore, the neuropathy observed in the brother II:5 and the son III:1 is not explained by a mutation of SPT alone, as neither p.A339V nor other mutations in SPT were found in these patients.

Nevertheless, all four male patients presenting with neurological abnormalities and even the unaffected daughter who carries the mutation had elevated concentrations of the neurotoxic 1-deoxySLs in their plasma. Increases in plasma concentration of these atypical 1-deoxySL species are an important hallmark of HSAN1 and have conclusively been linked to several mutations in SPTLC1 and SPTLC2 previously (9, 11, 17).

Biochemical analysis of the p.A339V mutation in our cell culture model initially supported its benignity, as neither the canonical activity of SPT was impaired, nor the alternative activity with alanine was increased. However, challenging the cells with high alanine concentrations created a different picture. Under these extreme conditions, the mutant showed a moderate

reduction of normal SPT activity and a moderate increase in 1-deoxySL formation. These changes were statistically significant, with $p<0.05$. Therefore, the new mutation p.A339V does affect the substrate preference and canonical activity of SPT, although in a much less pronounced way than the known British HSAN1-causing mutation p.C133W.

Along with p.A339V, at least two more variants of SPTLC1 are known (p.V144D and p.A352V) which do not obviously increase the activity of SPT with L-alanine in cells. In 2011, Rotthier and colleagues discussed this observation as the logical consequence of a more subtle effect of the p.A352V and p.V144D mutations on the biochemical properties of SPT, leading to a slower rate of 1-deoxySL formation (21). The p.A339V follows this pattern, exerting only minor effects on SPT activity. These effects might not be visible within 24 hours of culture but could nevertheless slowly increase the 1-deoxySL concentrations in the plasma of the patients. The severity of symptoms observed in this family is low to moderate, and the age at onset is rather late, after the 4th decade. This confirms the mild nature of the potential underlying mutation and fits the theory of a very slow accumulation of neurotoxic 1-deoxySLs.

However, the absence of neurological manifestations in the daughter causes another logical conflict with the hypothetical role of the p.A339V mutation as the cause of the disease. This contradiction might be explained by earlier reports observing gender specific differences in severity and penetrance of presented symptoms in hereditary neuropathies. In 2000, Auer-Grumbach et al reported the case of a large Austrian family (>100 members over 5 generations) affected by an autosomal dominant peripheral neuropathy. It was not fully clear whether the reported kinship was affected by HSAN1 or Charcot-Marie-Tooth type 2B (CMT2B). The clinical phenotype of the identified and suspected patients showed pronounced variability between the two extremes of “fully developed symptomatic until 30” and “no symptoms until older age”. In general, the authors observed more severe phenotypes in affected males within this family. Affected females noticed marked improvements of the disease during pregnancy and climacteric period. Taken together with the frequently observed onset of first symptoms after puberty, these observations strongly indicate the involvement of hormonal regulatory factors modifying the disease and attenuating its symptoms in affected women (23).

In 2006, Houlden and colleagues investigated eight British families with diagnosed HSAN1 caused by the mutation p.C133W in SPTLC1. They reported an average age of onset in women of 34 years, while men showed first symptoms at the age of 25. Moreover, females were shown to be less affected by several specific symptoms such as reduced sensory potentials in upper limbs and slowed motor nerve conduction velocities. These authors

suggested hormonal influences or sex-linked genetic factors to protect female carriers of the mutation from the symptoms of the disease (20).

Taken into account that the investigated p.A339V mutation in general causes only very mild effects on SPT, the daughter might not show HSAN1 symptoms due to the reported lower penetrance of the disease in women. The intermediately elevated levels of 1-deoxySLs in the plasma of the daughter support this theory, as their concentration was shown to correlate quite well with the severity of the disease (19). Last but not least, at 46 the daughter is still at an age where symptoms were absent in several reported cases (8 out of 35 (20)) that had late onset of the disease.

The augmented concentrations of 1-deoxySLs in the plasma of the oldest child (son III:1) and the brother of the index case however cannot be explained by altered phenotypical penetrance, as both men were tested negative for the p.A339V mutation. A closer look at the measured SL profiles of these two patients revealed generally elevated SL concentrations. Not only the neurotoxic 1-deoxySLs but also the normal C18 SLs and even the minor C20 SLs were present in higher concentrations compared to the other family members or healthy controls. This observation alone of course cannot explain the presence of 1-deoxySLs without involvement of a mutation in SPT, but points towards another mechanism affecting the regulation of SPT activity. A yet unknown second mutation might cause a deregulation of SPT activity, including its substrate preference, and thereby support the formation of 1-deoxySLs by the wild type enzyme. Recently several proteins that interact with SPT were reported to play a role in regulating its general activity and substrate preferences (24, 25). Those small interacting proteins represent promising candidates for further investigation of the underlying cause of the 1-deoxySLs in those two patients. Other potential candidates might be found in related metabolic pathways such as amino acid and fatty acid metabolism.

We cannot conclusively link the identified p.A339V mutation to the disease, although it presents with all the characteristic properties of a mild HSAN1-causing mutation. The absence of this mutation in two out of four affected males clearly contradicts its role as the sole cause for the neuropathy, and at the same time strongly argues for a second bystander mutation causing or at least contributing to the increased metabolization of L-alanine by SPT in these patients.

Further examination and whole exome sequencing of the family will be necessary to finally solve this riddle. The expected bystander mutation obviously causes an elevated activity of the wild type SPT complex with its alternative substrate L-alanine. This would be a gain of function in an unmutated SPT enzyme which has never been reported so far.

The identification of the causative mutation in this family therefore is of high importance not only for the diagnosis of the patients but also because it has a high potential to improve our understanding and knowledge of SPT regulation, the general pathomechanism, and the basis for the pronounced genetic and phenotypic variability observed in HSAN1.

4.8 References for chapter 4:

1. Rotthier, A., Baets, J., Timmerman, V., and Janssens, K. (2012) Mechanisms of disease in hereditary sensory and autonomic neuropathies. *Nat. Rev. Neurol.* **8**, 73–85

2. Edvardson, S., Cinnamon, Y., Jalas, C., Shaag, A., Maayan, C., Axelrod, F. B., and Elpeleg, O. (2012) Hereditary sensory autonomic neuropathy caused by a mutation in dystonin. *Ann. Neurol.* **71**, 569–72

3. Leipold, E., Liebmann, L., Korenke, G. C., Heinrich, T., Giesselmann, S., Baets, J., Ebbinghaus, M., Goral, R. O., Stödberg, T., Hennings, J. C., Bergmann, M., Altmüller, J., Thiele, H., Wetzel, A., Nürnberg, P., Timmerman, V., De Jonghe, P., Blum, R., Schaible, H.-G., Weis, J., Heinemann, S. H., Hübner, C. a, and Kurth, I. (2013) A *de novo* gain-of-function mutation in SCN11A causes loss of pain perception. *Nat. Genet.* **45**, 1399–404

4. Dyck, P. J. (1993) Neuronal atrophy and degeneration predominantly affecting peripheral sensory and autonomic neurons. in *Peripheral Neuropathy*, 3rd Editio (Dyck, P. J., Thomas, K. P., Griffin, J. W., Low, P. A., and Poduslo, J. F. eds), pp. 1065–1093, Saunders Philladelphia

5. Auer-Grumbach, M. (2008) Hereditary sensory neuropathy type I. *Orphanet J. Rare Dis.* **3**, 7

6. Auer-Grumbach, M., Bode, H., Pieber, T. R., Schabhüttl, M., Fischer, D., Seidl, R., Graf, E., Wieland, T., Schuh, R., Vacariu, G., Grill, F., Timmerman, V., Strom, T. M., and Hornemann, T. (2013) Mutations at Ser331 in the HSN type I gene SPTLC1 are associated with a distinct syndromic phenotype. *Eur. J. Med. Genet.* **56**, 266–9

7. Bejaoui, K., Wu, C., Scheffler, M. D., Haan, G., Ashby, P., Wu, L., de Jong, P., and Brown, R. H. (2001) SPTLC1 is mutated in hereditary sensory neuropathy, type 1. *Nat. Genet.* **27**, 261–2

8. Dawkins, J. L., Hulme, D. J., Brahmbhatt, S. B., Auer-Grumbach, M., and Nicholson, G. a (2001) Mutations in SPTLC1, encoding serine palmitoyltransferase, long chain base subunit-1, cause hereditary sensory neuropathy type I. *Nat. Genet.* **27**, 309–12

9. Murphy, S. M., Ernst, D., Wei, Y., Laurà, M., Liu, Y.-T., Polke, J., Blake, J., Winer, J., Houlden, H., Hornemann, T., and Reilly, M. M. (2013) Hereditary sensory and autonomic neuropathy type 1 (HSANI) caused by a novel mutation in SPTLC2. *Neurology*. **80**, 2106–11

10. Rotthier, A., Baets, J., De Vriendt, E., Jacobs, A., Auer-Grumbach, M., Lévy, N., Bonello-Palot, N., Kilic, S. S., Weis, J., Nascimento, A., Swinkels, M., Kruyt, M. C., Jordanova, A., De Jonghe, P., and Timmerman, V. (2009) Genes for hereditary sensory and autonomic neuropathies: a genotype-phenotype correlation. *Brain*. **132**, 2699–711

11. Rotthier, A., Auer-Grumbach, M., Janssens, K., Baets, J., Penno, A., Almeida-Souza, L., Van Hoof, K., Jacobs, A., De Vriendt, E., Schlotter-Weigel, B., Löscher, W., Vondráček, P., Seeman, P., De Jonghe, P., Van Dijck, P., Jordanova, A., Hornemann, T., and Timmerman, V. (2010) Mutations in the SPTLC2 subunit of serine palmitoyltransferase cause hereditary sensory and autonomic neuropathy type I. *Am. J. Hum. Genet.* **87**, 513–22

12. Rautenstrauss, B., Neitzel, B., Muench, C., Haas, J., and Holinski-Feder, E. (2009) LATE ONSET HEREDITARY SENSORY NEUROPATHY TYPE 1 (HSN1) CAUSED BY A NOVEL P.C133R MISSENSE MUTATION IN SPTLC1 Würzburg, Germany. in *2009 Meeting of the Peripheral Nerve Society July 4-8, 2009*, p. 290 of 381

13. Ernst, D. (2013) Regulation of Deoxy-Sphingolipids and Their Role in Disease. *PhD Thesis*

14. Ernst, D., Murphy, S. M., Sathiyanadan, K., Wei, Y., Othman, A., Laura, M., Donaghy, M., Houlden, H., Reilly, M. M., and Hornemann, T. (2015) Novel HSAN1 Mutation in Serine Palmitoyltransferase Resides at a Putative Phosphorylation Site That Is Involved in Regulating Substrate Specificity. *NeuroMolecular Med.* **17**, 47–57

15. Davidson, G. L., Murphy, S. M., Polke, J. M., Laura, M., Salih, M. A. M., Muntoni, F., Blake, J., Brandner, S., Davies, N., Horvath, R., Price, S., Donaghy, M., Roberts, M., Foulds, N., Ramdharry, G., Soler, D., Lunn, M. P., Manji, H., Davis, M. B., Houlden, H., and Reilly, M. M. (2012) Frequency of mutations in the genes associated with hereditary sensory and autonomic neuropathy in a UK cohort. *J. Neurol.* **259**, 1673–1685

16. Hanada, K. (2003) Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids*. **1632**, 16–30

17. Penno, A., Reilly, M. M., Houlden, H., Laurá, M., Rentsch, K., Niederkofler, V., Stoeckli, E. T., Nicholson, G., Eichler, F., Brown, R. H., von Eckardstein, A., and Hornemann, T. (2010) Hereditary sensory neuropathy type 1 is caused by the accumulation of two neurotoxic sphingolipids. *J. Biol. Chem.* **285**, 11178–87

18. Garofalo, K., Penno, A., and Schmidt, B. (2011) Oral l-serine supplementation reduces production of neurotoxic deoxysphingolipids in mice and humans with hereditary sensory autonomic neuropathy type 1. *J. Clin. …*. 10.1172/JCI57549.)

19. Laurá, M., Murphy, S. M., Hornemann, T., Bode, H., Polke, J., Blake, J., Houlden, H., and Reilly, M. M. (2012) P42 Hereditary sensory neuropathy type 1: correlation of severity and plasma atypical deoxy-sphyngoid bases. *Neuromuscul. Disord.* **22**, S18

20. Houlden, H., King, R., Blake, J., Groves, M., Love, S., Woodward, C., Hammans, S., Nicoll, J., Lennox, G., O'Donovan, D. G., Gabriel, C., Thomas, P. K., and Reilly, M. M. (2006) Clinical, pathological and genetic characterization of hereditary sensory and autonomic neuropathy type 1 (HSAN I). *Brain*. **129**, 411–25

21. Rotthier, A., Penno, A., Rautenstrauss, B., Auer-Grumbach, M., Stettner, G. M., Asselbergh, B., Van Hoof, K., Sticht, H., Lévy, N., Timmerman, V., Hornemann, T., and Janssens, K. (2011) Characterization of two mutations in the SPTLC1 subunit of serine palmitoyltransferase associated with hereditary sensory and autonomic neuropathy type I. *Hum. Mutat.* **32**, E2211–25

22. Hornemann, T., Richard, S., Rütti, M. F., Wei, Y., von Eckardstein, A., Serine-palmitoyltransferase, M., Ru, M. F., and Eckardstein, A. Von (2006) Cloning and initial characterization of a new subunit for mammalian serine-palmitoyltransferase. *J. Biol. Chem.* **281**, 37275–81

23. Auer-Grumbach, M., Wagner, K., Timmerman, V., De Jonghe, P., and Hartung, H.-P. (2000) Ulcero-mutilating neuropathy in an Austrian kinship without linkage to hereditary motor and sensory neuropathy IIB and hereditary sensory neuropathy I loci. *Neurology*. **54**, 45–45

24. Han, G., Gupta, S. D., Gable, K., Niranjanakumari, S., Moitra, P., Eichler, F., Brown, R. H., Harmon, J. M., and Dunn, T. M. (2009) Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 8186–91

25. Breslow, D. K., Collins, S. R., Bodenmiller, B., Aebersold, R., Simons, K., Shevchenko, A., Ejsing, C. S., and Weissman, J. S. (2010) Orm family proteins mediate sphingolipid homeostasis. *Nature*. **463**, 1048–53

5 Biochemical classification of SPT mutations causing HSAN1 reveals distinct clusters and genotype severity correlation.

Manuscript accepted for publication in Human Molecular Genetics

Bode, Heiko; University Hospital Zurich, Clinical Chemistry
Bourquin, Florence; University Zurich, Institute of Biochemistry
Suriyanarayanan, Saranya; University Hospital Zurich, Clinical Chemistry
Wei, Yu; University Hospital Zurich, Clinical Chemistry
Alecu, Irina; University Hospital Zurich, Clinical Chemistry
Othman, Alaa; University Hospital Zurich, Clinical Chemistry
von Eckardstein, Arnold; University Hospital Zurich, Clinical Chemistry
Hornemann, Thorsten; University Hospital Zurich, Clinical Chemistry

This Chapter was accepted for publication in "Human Molecular Genetics" on 12th of December 2015. The chapter was shortened and formatted by Thorsten Hornemann who also changed the title to "HSAN1 mutations in serine palmitoyltransferase reveal a close structure-function-phenotype relationship". An additional section on homology modelling and structural analysis of the mutated subunits, based on the work of Florence Bourquin, Saranya Suriyanarayanan and Yu Wei, and another small section on the activity of HSAN1 mutants in LYB cells, based on my own work and data, were added to the submitted version of this chapter by Thorsten Hornemann.

My contribution to this chapter and the resulting publication covers the cloning of eleven mutations into our expression system, transfection and generation of stable cell lines, cell culture, planning and execution of the experiments, lipid extraction from harvested cells (and plasma samples), measurement of the sphingoid base backbones by LC-MS, quantification and data analysis and writing of this chapter as base for the manuscript.

I had some support during the harvesting of the cells from Irina Alecu and Alaa Othman supported me during the statistical analysis (PCA) of the generated dataset.

5.1 Abstract:

5.1.1 Background:

Hereditary sensory and autonomic neuropathy type 1 (HSAN1) is an autosomal dominant inherited mutilating peripheral neuropathy, characterized by the degeneration of peripheral nerves. Several mutations in serine palmitoyltransferase (SPT) have been conclusively associated with this rare neuropathy. Some of these mutations cause a shift in substrate preference of SPT, raising its activity with L-alanine and glycine which results in pathological production of neurotoxic 1-deoxysphingolipids (1-deoxySL).

5.1.2 Primary objective of the study:

In this study we compare the biochemical properties of eleven missense mutations in the SPTLC1 and six mutations in the SPTLC2 subunit of SPT. We aim to provide a comprehensive dataset on the individual properties of the different mutations. We search for common characteristics and potential phenotype - genotype correlations within the different mutations. Furthermore we report a new mutation found in a German patient suffering from peripheral neuropathy of unknown cause.

5.1.3 Methods:

All previously reported mutations of SPTLC1 and SPTLC2 were introduced by site directed mutagenesis and stably expressed in HEK293 cells. We used stable isotope metabolic labeling with deuterated amino acids and LC-MS to analyze the individual sphingoid base (SB) profiles. The obtained SB fingerprints reflect the catalytic activity and substrate preference of SPT under the particular assay conditions.

5.1.4 Results:

We demonstrated that none of the investigated mutations in SPTLC1 or SPTLC2 reduced the canonical intracellular activity with L-serine in physiological conditions. In three cases we observed an increased canonical activity under these conditions.

However, in the presence of the Ceramide Synthase (CerS) inhibitor Fumonisin B1 we observed significant reductions in the canonical activity in eleven mutations.

Increased synthesis of the atypical 1-deoxySL *in vitro* was associated with totally eight mutations and occured independent of FB1. By principal component analysis (PCA), we observed a clustering of three distinct groups of mutations indicating a clear genotype-phentotype relationship between the type of mutation and the clinical symptoms in the affected patients.

5.1.5 Conclusions:

The clustering of the mutants reflects the symptomatic and genetic heterogeneity of HSAN1 and allows to predict the course of the disease based on the biochemical properties of the underlying mutations in SPT. The use of easily reproducible and standardized tests will facilitate the fast and easy amendment of future mutations to the presented dataset.

5.2 Introduction:

The term hereditary sensory and autonomic neuropathy (HSAN) represents a group of neurologic disorders affecting the peripheral nervous system with high genotypic and phenotypic variability. HSAN has been further subdivided into seven classes with distinct phenotypes (1–3). The only autosomal dominant trait HSAN type one (HSAN1) can be discriminated from the recessive inherited traits (HSAN2-7) by its delayed onset between 2nd and 5th decade of life, the slowly progressive course and the predominant involvement of the lower limbs (1, 4).

HSAN1 is a mutilating, axonal neuropathy typically characterized by prominent slowly progressive sensory loss, formation of perforating ulcers at the feet and an onset between 2nd and 5th decade(1, 4, 5). The clinical symptoms include atrophy and demyelination in the cauda equine, thinning of the sciatic and ulnar nerves, atrophy of the dorsal root ganglia and significant loss of myelinated fibers in several afferent peripheral nerves. Degeneration of axons in the dorsal root therefore was initially supposed to cause the neurologic impairments (6).

Mutations in five different genes were identified and associated with HSAN1. Amino acid exchanges in the sequences of Atlastin1, RAB7A and DNMT1 are known to cause a neuropathy with HSAN1-like pathology (7)

The majority of HSAN1 causing mutations however were reported in the sequence of two subunits of serine palmitoyltransferase (SPT).

Serine palmitoyltransferase (SPT) is a PLP-dependent α-oxoamine synthase (POAS), located at the outer membrane of the endoplasmic reticulum. SPT catalyzes the condensation of palmitoyl-CoA and Serine to 3-ketodihydrosphinganine (KDS), the first and rate limiting step of sphingolipid *de novo* synthesis. Mammalian SPT was considered to be a heterodimer of the SPT subunits, serine palmitoyltransferase long chain base subunit 1 and 2 (SPTLC1 and SPTLC2). SPTLC2 exclusively contains a PLP binding domain, which is indispensable for the covalent binding of the cofactor PLP and the catalytic activity of the enzyme (8). A third subunit SPTLC3 was reported, which also contains the PLP binding motif, suggesting a similar function as SPTLC2 (9). Expression of SPTLC3 increased the metabolization of lauroyl- and myristoyl-CoA thereby increasing the formation of short chain (C14 and C16) sphingoid bases (10). All three SPT subunits presumably interact and form a hetero-octameric SPT enzyme complex with four active sites (11).

In total thirteen mutations of SPTLC1 and six of SPTLC2 are known (for an overview see Table 2 and Table 3 and Figure 5-1).

Subunit	Locus	Background	Included in this study	Reference
SPTLC1	p.C133R	Associated with HSAN1 (isolated case of late onset peripheral neuropathy)	No	(12)
	p.C133W	Associated with HSAN1	Yes	(13, 14)
	p.C133Y	Associated with HSAN1	Yes	(13, 14)
	p.V144D	Associated with HSAN1	Yes	(14)
	p.R151L	Polymorphism identified in an HSAN1 patient	Yes	(15)
	p.R239W	Genomic screening for breast cancer associated mutations	Yes	(16)
	p.G246R	This mutation causes the total disruption of SPT activity in CHO cells	Yes	(17, 18)
	p.A310G	Published but not conclusively associated mutation	No	(19)
	p.A339V	Unpublished mutation found in a German family	Yes	This work
	p.S331F	Associated with HSAN1	Yes	(20)
	p.S331Y	Associated with HSAN1	Yes	(21)
	p.A352V	Associated with HSAN1	Yes	(20)
	p.G387A	Confirmed non-causing mutation	Yes	(22, 23)

Table 2 Overview on all known mutations of SPTLC1

Subunit	Locus	Background	Included in this study	Reference
SPTLC2	p.A182P	Associated with HSAN1	Yes	(24)
	p.V359M	Associated with HSAN1	Yes	(25)
	p.G382V	Associated with HSAN1	Yes	(25)
	p.S384F	Associated with HSAN1	Yes	(26, 27)
	p.T409M	Isolated case not conclusively associated with HSAN1	Yes	Not published
	p.I504F	Associated with HSAN1	Yes	(25)

Table 3 Overview on all known mutations of SPTLC2

The HSAN1-associated mutations cause a shift in the amino acid preference of SPT resulting in increased activity of the enzyme with the two alternative amino acid substrates L-alanine and glycine. Metabolization of L-alanine and glycine results in increased formation of 1-deoxysphinganine (1-deoxySA) and 1-desoxymethylsphinganine (1-desoxyMeSa) respectively. The subsequent N-acylation of 1-deoxysphingoid bases (1-deoxySB) results in the formation of a new class of atypical Sphingolipids named 1-deoxysphingolipids (1-deoxySL). Increased concentrations of 1-deoxySL were found in HEK293 cells expressing the mutated SPT but also in lymphoblasts and plasma of HSAN1 patients (28).

The lack of the C_1 hydroxyl group impedes further processing of the 1-deoxySL by attaching headgroups at this position. Therefore, 1-deoxySL cannot be processed to complex sphingolipids like sphingomyelins (head groups phosphocholine) or glycosphingolipids (head groups sugar moieties, hexoses) respectively. Furthermore the 1-deoxySL can also not be degraded by the canonical catabolic pathway of sphingolipdis, which requires the phosphorylation of C_1 to build the catabolic intermediate sphingosine-1-phosphate (28).

The 1-deoxySB were shown to be cytotoxic on the prostate cancer cell line DU-145 (29). Addition of 1-deoxySB to vero cells reduces their proliferation rate and causes cytoskeletal changes by the disassembly of actin stress fibers (30).

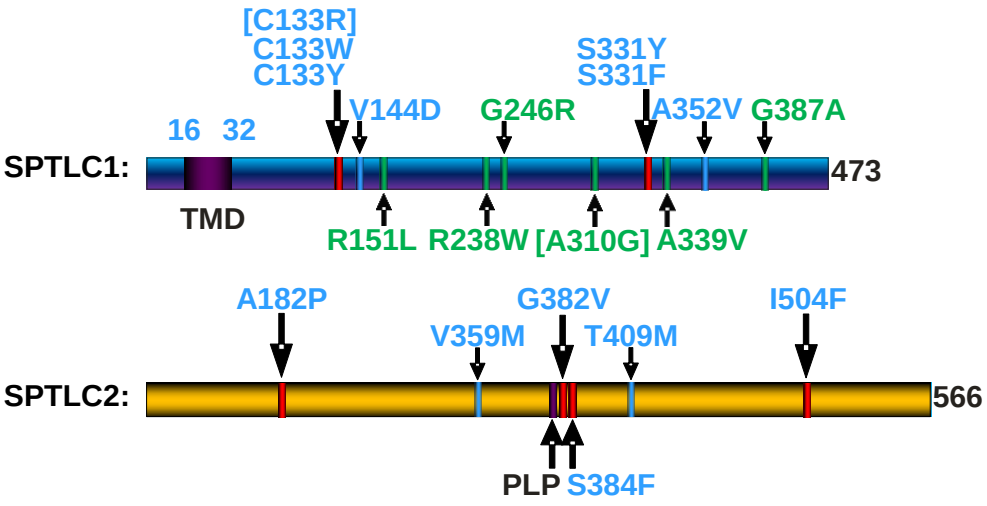
Sensory and motor neuropathy together with neuropathic pain and even central neurotoxicity caused by the free base 1-deoxysphinganine were reported as adverse effects in two phase I studies, when investigating the potential of 1-deoxySA as an experimental anticancer drug. Neurotoxicity was seen in cultured primary chicken embryonic neurons (28, 31, 32). Therefore we concluded that HSAN1 is not caused by a loss but a gain of function which

results in an increased formation of neurotoxic 1-deoxysphingolipids within the peripheral neurons and the slow decay of axonal structures (28).

Elevated levels of 1-deoxySL in plasma were consistently found in all HSAN1 patients bearing one of the above introduced disease-causing mutations in SPT. Elevated 1-deoxySL therefore represent a reliable biomarker and important biochemical hallmark of HSAN1.

Besides its obvious genotypic variability, HSAN1 is also characterized by profound phenotypical variability with a strong correlation between severity of the symptoms and the underlying genotype. Some mutations in SPT such as SPTLC1 p.V144D are associated with rather mild symptoms whereas other variants (SPTLC1 p.S331F and p.S331Y and SPTLC2 p.I504F) give rise to a severe phenotype characterized by early onset, anhydrosis and pronounced muscle waste. Additional symptoms such as growth and in one case even mental retardation, ocular manifestations and pneumological complications are present exclusively for those three mutants (20, 21, 25, 33–35).

A recently reported p.A182P mutation in SPTLC2 causes an earlier onset of the disease and severe muscle wasting in upper and lower limbs but additional symptoms such as involvement of vision and autonomous dysfunction like sweating disturbances are absent (24).



.

Figure 5-1: Graphical overview of all known mutations of SPTLC1 and SPTLC2. Mutations printed in blue were conclusively associated with HSAN1, mutations in green were found independent of HSAN1 or not yet reported to cause the disease. Mutations which cause increased activity of SPT with L-alanine *in vitro* are indicated by red bars. The two mutations in parentheses were not included in this study.

With the recent progress in diagnostic sequencing techniques, fast and cheap identification of genomic mutations became possible and resulted in discovery of at least one new mutation in SPT per year. This reflects the marked genotypic heterogeneity of this disease and SPT´s general importance for preservation of peripheral sensory nerves. The hallmark characteristics of HSAN1 are conserved in all patients, however severity and occurrence of additional clinical symptoms like congenital onset, cataract formation and growth retardation present a high variability with strong correlation to the underlying mutation.

Elevated 1-deoxySL concentrations in plasma therefore represent the only reliable and constant hallmark for all HSAN1-associated mutations.

In this study we will provide a comprehensive comparison of the biochemical characteristics of all yet reported SPT mutations. We applied a set of uniform metabolic labeling conditions to determine these parameters in a comparable and reproducible manner.

We observed a direct association between the biochemical properties of the mutated SPT subunits and their clinical phenotype.

Furthermore we included one unpublished, putative HSAN1-causing mutations in SPTLC1 and SPTLC2 respectively in the dataset and another one, which was published only recently. The mutation (SPTLC1 p.A339V) was found in a German patient aged 72, suffering from peripheral neuropathy with unknown cause. Another only recently reported mutation (SPTLC2 p.S384F) was found in two unrelated British families (26, 27). Both mutations were included to examine their biochemical properties in direct comparison with all the other HSAN1 mutations.

5.3 Materials and Methods:

5.3.1 Cloning of SPT mutations:

All constructs were amplified out of a human cDNA library (Matchmaker cDNA Library Muscle/Brain, Clontech, Mountain View, CA) by PCR using appropriate primers and the Long-Expand Mix (Roche Applied Science). The following primers (Table 4) were used for initial cloning of the expression plasmids (9):

Oligo name	Sequence
SPTLC1fw	5'-caccatggcgaccgccacggagcagtgggttc-3'
SPTLC1rv	5'-gagcaggacggcctgggctacctcc-3'
SPTLC2fw	5'-caccatgcggccggagcccggaggctgctgctgc-3'
SPTLC2rv	5'-gtcttctgtttcttcatacgtcgtctcg-3'

Table 4 primers for amplification of human serine palmitoyltransferase subunit 1 and 2 out of cDNA Library (9)

Mutations were inserted into the expression vectors SPTLC1 or SPTLC2 respectively in pcDNA™3.1/V5-His TOPO® (Invitrogen) (9) by site directed mutagenesis. For the amplification of the expression vectors, we used Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) and the following primer pairs (see Table 5).

Oligo name	Sequence
SPTLC1_C133Wfw	5′-ggggacccagaggattttatggcacattt gatgttc-3′
SPTLC1_C133Wrv	5′-aggtacccacgcca tacttctttagagatgctaaagc-3′
SPTLC1_C133Yfw	5′-atggtcctcgaggattttatggcacattt gatgttc-3′
SPTLC1_C133Yrv	5′-aggtacccacgcca tacttctttagagatgctaaagc-3′
SPTLC1_V144Dfw	5′-gtggacccagaggattttatggcacattt gatgatcatttggatttggaagaccg-3′
SPTLC1_V144Drv	5′-aggtacccacgcca tacttctttagagatgctaaagc-3′
SPTLC1_R151L_for	5´-Ttcatttggatttggaagacctcctggcaaaatttatgaagac-3´
SPTLC1_R151L_rev	5´-Gtcttcataaattttgccaggaggtcttccaaatccaaatgaa-3´
SPTLC1_R239W_for	5´-Cctcgcaaggctcgtgtaacttggcgtttcatt-3´

SPTLC1_R239W_rev	5´-Aatgaaacgccaagttacacgagccttgcgagg-3´
SPTLC1_G246R_for	5´-Cggcgtttcattgtagtagaacgattgtatatgaatactggaa-3´
SPTLC1_G246R_rev	5´-Ttccagtattcatatacaatcgttctactacaatgaaacgccg-3´
SPTLC1_S331F_for	5´-Tgtaattgaccatcagcgacttttcggccagggata-3´
SPTLC1_S331F_rev	5´-Tatccctggccgaaaagtcgctgatggtcaattaca-3´
SPTLC1_S331Y_for	5´-Tgtaattgaccatcagcgactttacggccagggata-3´
SPTLC1_S331Y_rev	5´-tatccctggccgtaaagtcgctgatggtcaattaca-3´
SPTLC1_A339Vfw	5´-gggatacTGCTTTTCAGtaagcTTACCTcccctgttagc-3´
SPTLC1_A339Vrv	5´-gctaacaggggAGGTAAgcttaCTGAAAAGCAgtatccc-3´
SPTLC1_A352V_for	5´-Tgctgcagcaattgaggtcctcaacatcatggaag-3´
SPTLC1_A352V_rev	5´-Cttccatgatgttgaggacctcaattgctgcagca-3´
SPTLC1_G387Afw	5′-aagtggtggcggagtccctttctccagccttt cacct-3′
SPTLC1_G387Arv	5′-ttaatccagatatcccttgtaaagctttat gaatttgtcc-3′
SPTLC2_A182P_fw	5´-CAACTATCTTGGATTTCCCCGGAATACTGGATCATG-3´
SPTLC2_A182P_rv	5´-CATGATCCAGTATTCCGGGGAAATCCAAGATAGTTG-3´
SPTLC2_V359M_for	5´-Ccacaggccggggtatggtggagtac-3´
SPTLC2_V359M_rev	5´-Gtactccaccataccccggcctgtgg-3´
SPTLC2_G382V_for	5´-Gaacgttcacaaagagttttgttgcttctggaggatatattgg-3´
SPTLC2_G382V_rev	5´-Ccaatatatcctccagaagcaacaaaactctttgtgaacgttc-3´
SPTLC2_S384F_fw	5'-CAAAGAGTTTTGGTGCTTTCGGAGGATATATTGGAGGC-3'
SPTLC2_S384F_rv	5'-GCCTCCAATATATCCTCCGAAAGCACCAAAACTCTTTG-3'
SPTLC2_T409M_for	5´-Tagtgcagtgtatgccatgtcattgtcacctcctg-3´
SPTLC2_T409M_rev	5´-Caggaggtgacaatgacatggcatacactgcacta-3´
SPTLC2_I504F_for	5´-Ttcctgccaccccaatttttgagtccagagcc-3´
SPTLC2_I504F_rev	5´-Ggctctggactcaaaaattggggtggcaggaa-3´

Table 5 Primers for site directed introduction of mutations into the pcDNA3.1-SPTLC1 and pcDNA3.1-SPTLC2 expression vectors

PCR products were transformed into chemical competent Top10 *E. coli* bacteria. Single colonies from selection plates (LB+Amp) were incubated overnight and vectors were extracted and purified with the QIAprep Spin Miniprep Kit (Qiagen).

Correct insertion of the mutation was confirmed by sequencing of the obtained expression vectors, using BigDye Terminator v3.0 cycle sequencing kit and the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

5.3.2 Transfection of constructs with Lipofectamine 2000 (Invitrogen)

HEK293 cells were grown in 6-well dishes to 80% confluency.
Four µg of the plasmid were mixed with 50µl serumfree Opti-MEM medium (Life Technologies). Another 50µl Opti-MEM were incubated together with 10µl of Lipofectamine 2000 (Life Technologies) for 5min. Both mixtures were combined and incubated at roomtemperature. After 15min 900µl of normal growthmedium without antibiotics (DMEM + 10%FCS) were added to the transfection mix.
The HEK293 cells were washed twice with PBS. Afterwards one ml of the Plasmid-Lipofectamine mix was added to the cells and they were incubated for at least 5h at 37°C and 5%CO in a humidified incubator (Hera Cell, Heraeus). After this initial incubation another 1200µl growthmedium without antibiotics were added. Two days later, the transfection medium was exchanged for selection medium containing P/S and the 400µg/l G418 (Gibco).

After transfection the cells were passaged for min. 3 more times in selection medium to establish a stable expressing clone of transgenic cells. Stable expressing cells were aliquoted and frozen in $N_{2\ liq}$ in selection medium containing 5% of DMSO (Sigma).

After thawing of the cells, they were passaged at least two more times before the experiment

Expression of the .transfected plasmids was confirmed prior to the experiment by western blotting: 25µg cellular protein was separated on a 12% SDS Page and detected by a combination of His- and V5-tag antibodies (both from Serotec). GAPDH was used as the loading control (lower band) (see Figure 5-2 A and B and Figure 5-3).

5.3.3 Cell culture:

HEK293 cells were obtained from the ATCC and cultured in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal calf serum (Fisher Scientific FSA15-043) and penicillin/streptomycin (100 units per ml/0.1 mg per ml, Sigma). (28)

HEK293 cells transfected with the pcDNA3.1 expression constructs were incubated in selection medium: DMEM (Sigma) + 10% FCS (Fisher Scientific FSA15-043) + P/S (100 units per ml/0.1 mg per ml, Sigma) and G418 (400µg/ml, Gibco).

All Cells were incubated @ 37°C with 5%CO_2 in a humidified incubator (Hera Cell, Heraeus)

5.3.4 General setup of the assays:

250 000 cells/well were seeded in 2ml fresh medium in 6-well plates (BD Falcon) and incubated for two more days until ~80-90%confluency. After 24h of incubation, the selection medium was exchanged for L-serine and L-alanine-free DMEM (Genaxxon Bioscience, Ulm, Germany), containing 10% FCS and P/S and 400µg/ml G418. After this medium change, cells were incubated without L-serin and L-alanine for 2 more hours @37°C and 5% CO_2.

For the standard d3-labeling assay, we added the labeled substrates 2mM (2,3,3,3)-d4-L-alanine and 1mM d3-N_{15}1-L-serine to the cells.

For the fumonisin B1 (FB1) assay as described by Zitomer (29). We added FB1 (10mg/ml in 100% EtOH, Sigma Aldrich) to a final concentration of 35µM together with the deuterated substrates, to the cells.

After another 24 hours of incubation, the cells were harvested in 1ml cold PBS, cells were counted (Beckman Coulter Z2), pelleted at 800rcf and 4°C in an Eppendorf 5415 table top centrifuge (Eppendorf, Hamburg, Germany) and stored frozen at -20°C until further processing.

5.3.5 Acid and Base Hydrolysis:

The extraction and HPLC conditions used in our experiments were adapted from the "Sample protocol for the long method" by Riley and colleagues (36).

Cell pellets were resuspended in 100µl phosphate buffered saline (PBS), 500µl MeOH (Honeywell), including the deuterated internal standards d7-SA and d7-SO (200pmol each, Avanti Polar lipds, Alabaster CA) were added and lipids were extracted for 1h under constant agitation with 1400rpm @ 37°C on a Eppendorf Thermomixer Comfort (Eppendorf, Hamburg, Germany). Precipitated protein was removed by centrifugation for 5min @ 16100rcf and 22°C in an Eppendorf 5415 table top centrifuge (Eppendorf, Hamburg, Germany). 500µl of the supernatant were transferred to fresh tubes and 75µl of HCl (32%, Sigma) were added. The extracted lipids were hydrolyzed for min. 12hours @ 65°C. Acid hydrolysis was stopped next day by addition of 100µl KOH [10M], lipid extraction was introduced by adding 125µl $CHCl_3$. The separation of organic and inorganic phases was started by addition of another 500µl $CHCl_3$, followed by 100µl NH_4OH [2N] and finally 500µl alkaline H_2O. Samples were mixed by vigorous vortexing after every addition. Phase separation was leveraged by centrifugation @ 16100rcf and 22°C for another 5min. Upper polar phase was removed, using Pasteur pipette and vacuum pump (BVC21, Vacuubrand, Theilingen, Schweiz)). Chloroform phase was washed two more times with 1ml alkaline H_2O. The remaining $CHCl_3$

phase was evaporated under constant N_2-flow in Techne Sample Concentrator (Bibby Scientific Ltd, Staffordshire, UK) for ~20min. Dried lipids were stored at -20°C.

5.3.6 Derivatization and LC-MS:

Dried Lipids were redissolved in 75µl of derivatization Mix (56.7% MeOH, 33.3% EtOH, 10% H_2O) and derivatized with 5µl of OPA working solution (990µl boric acid [3%] + 10µl o-Phthalaldehyde [50mg/ml in EtOH] + 0.5µl 2-Mercaptoethanol).

Samples were separated on a reverse phase C18 column (Uptispere 120 Å, 5µm, 125x2 mm, Interchim, France) at a flowrate of 400µl/min. Buffers were A: 50% MeOH + 5 mM Ammonium Acetate (Sigma) and B: 100%MeOH.

Mass spectra were recorded in full scan positive ion mode by a triple quad detector (TSQ quantum ultra, Thermo scientific).

5.3.7 Statistics

Significance of the observed variations was tested by one way ANOVA followed by Dunnett's Multiple comparison Test, using the software GraphPad Prism 5.
The Principal component analysis (see Figure 5-10 and Figure 5-11) was done in SIMCA-P+ (Umetrics).

5.4 Results:

5.4.1 Overexpression of SPT mutations in HEK293 cells

The base for this analysis was a cell culture model with 21 different cell lines in total (17 mutants, 3 control constructs and the HEK293 wild type). As shown in Figure 5-2 A all SPTLC1 constructs were expressed and could be detected on the blot right before the experiments. For unknown reasons the signals from the mutants p.A339V and p.A352V were weaker compared to other SPTLC1 constructs, or the loading control (GAPDH). As shown in Figure 5-2 B the constructs were expressed and the ratios between mutant and GAPDH bands were comparable for all SPTLC2 mutants.

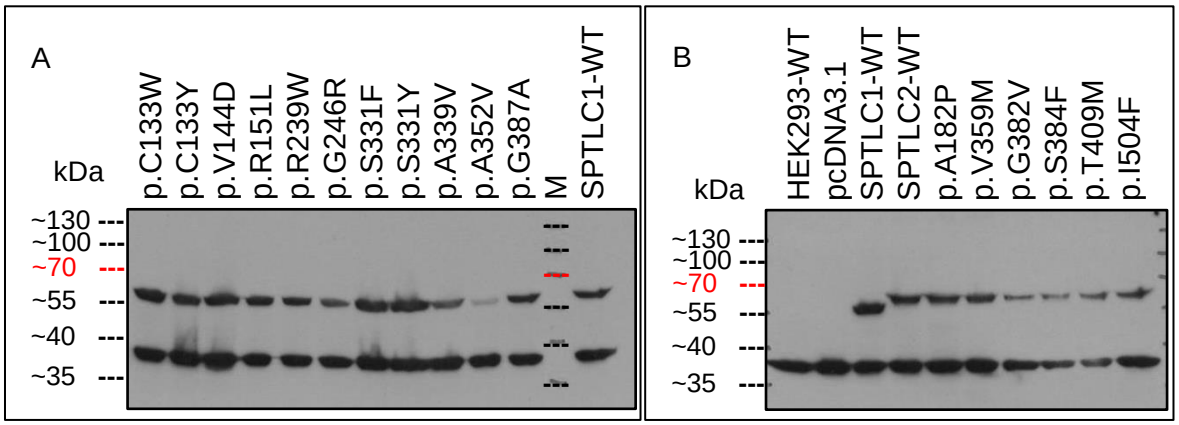


Figure 5-2 Protein expression control after thawing of the stable transfected HEK293 cells. The proteins were separated on a 12% SDS Page and detected by a combination of His- and V5-tag antibodies (both from Serotec, higher bands). GAPDH was used as the loading control (lower bands). A shows the analyzed mutants of SPTLC1, B shows un- or vector-transfected controls and mutants affecting the second subunit SPTLC2.

For the later activity experiments, using high doses (10mM) of L-alanine and L-serine a new batch of cells was thawed and expression of the constructs was confirmed as described above. Again all mutants were expressed (see Figure 5-3).

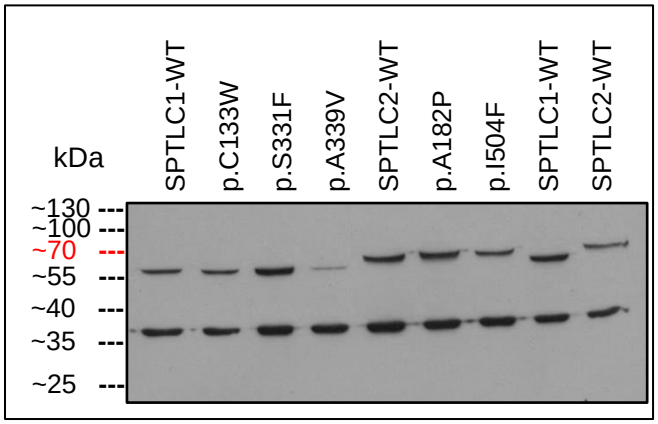


Figure 5-3 Protein expression control after thawing of the second batch of stable transfected HEK293 cells. The proteins were separated on a 12% SDS Page and detected by a combination of His- and V5-tag antibodies (both from Serotec, higher bands). GAPDH was used as the loading control (lower bands).

5.4.2 Effects of the different mutations on canonical SPT reaction.

Intracellular sphingolipid *de novo* synthesis was measured by adding stable isotope labeled d3 or d3-N_{15}1-L-serine (depending on the assay) and d4-L-alanine to the cell culture. After incubation for 24h the incorporation of the labelled amino acids into the *de novo* formed sphingoid bases was quantified by LC-MS. Concentrations of the sphingoid backbones were determined by subjecting the total extracted sphingolipids to acid and base hydrolysis. The concentrations of labeled (d3- or d2-) C18SA and C18SO, (d3-) 1-deoxySA and 1-deoxySO and (d3- or d2-) C20SA and C20SO were analyzed and corrected for isotopic distribution. The concentration of isotope labelled sphingoid bases reflected the amounts of de-novo formed sphingolipids and deoxy-sphingolipids within 24h.

Mutations in SPT do not reduce the intracellular canonical SPT activity

The isolated overexpression of SPTLC1 did not increase SPT activity in comparison to the empty vector control (sample pcDNA3.1), whereas the overexpression of SPTLC2 resulted in a 2-fold increased SPT activity (see Figure 5-4). This indicates that SPTLC2 rather than SPTLC1 is the catalytically active subunit which is further supported by the fact that only SPTLC2 but not SPTLC1 contains the consensus sequence for a PLP binding site. It also indicates that the expression level of SPTLC2 is the limiting factor for the formation of an active SPT complex.

Although a reduced *in-vitro* activity was reported for several of the here reported mutations (37–39) none of the SPTLC1 and SPTLC2 mutants showed a significantly reduced incorporation of d3-serine in comparison to wild type (SPTLC1 and SPTLC2 respectively) expressing cells (see Figure 5-4).

In contrast three of the tested mutations showed significantly increased incorporation of deuterated serine compared to the wild type. The exchange of serine to phenylalanine or tyrosine at SPTLC1position 331 (p.S331F and p.S331Y) increased the canonical activity of SPT in comparison to the wild type enzyme. A similar effect was observed by an exchange of isoleucine against phenylalanine in SPTLC2 at position 504 (p.I504F) which resulted in a doubling of SPT activity.

This pattern changed when the incorporation of d3-N_{15}1-L-serine was analyzed in the presence of the mycotoxin fumonisin B1 (FB1). FB1 is a potent inhibitor of ceramide synthase (CerS) which catalyzes the N-acylation of the free sphingoid base sphinganine to dihydroceramide downstream of SPT. CerS also catalyzes the reacylation of free sphingosine, derived from the recycling of complex sphingolipids.

The addition of FB1 blocks the *de novo* formation of SL downstream products and causes an accumulation of the CerS substrate sphinganine (40). Besides it was reported that the presence of FB1 leads to an increased formation of 1-deoxysphinganine by the wild type SPT (29) although the underlying mechanism is not fully understood yet.

In the presence of FB1 we observed significant differences in the canonical activity between the mutants (see Figure 5-5). While the SPTLC1 variants p.V144D, p.A339V and p.G387A showed a similar activity as the SPTLC1 or empty vector expressing cells, the incorporation of d3-N_{15}1-L-serine was significantly reduced in cells expressing the p.C133W, p.C133Y, p.R239W, p.G246R, and p.A352V mutations. Interestingly the two SPTLC1 mutations which showed an increased canonical activity under untreated conditions, presented a moderate but non-significant reduction (p.S331Y) and a significant reduced canonical activity (p.S331F) in the presence of FB1. Similar observations were made for the SPTLC2 mutations. For the p.A182P, p.V359M, p.G382V and p.S382F mutation we observed reduced activity whereas SPT activity in p.T409M expressing cells was not altered in comparison to SPTLC2 wild type expressing cells. In contrast to the previous assay and analogous to the SPTLC1 p.S331F and p.S331Y mutations the p.I504F mutation did not show increased but a significantly decreased canonical activity in the presence of FB1.

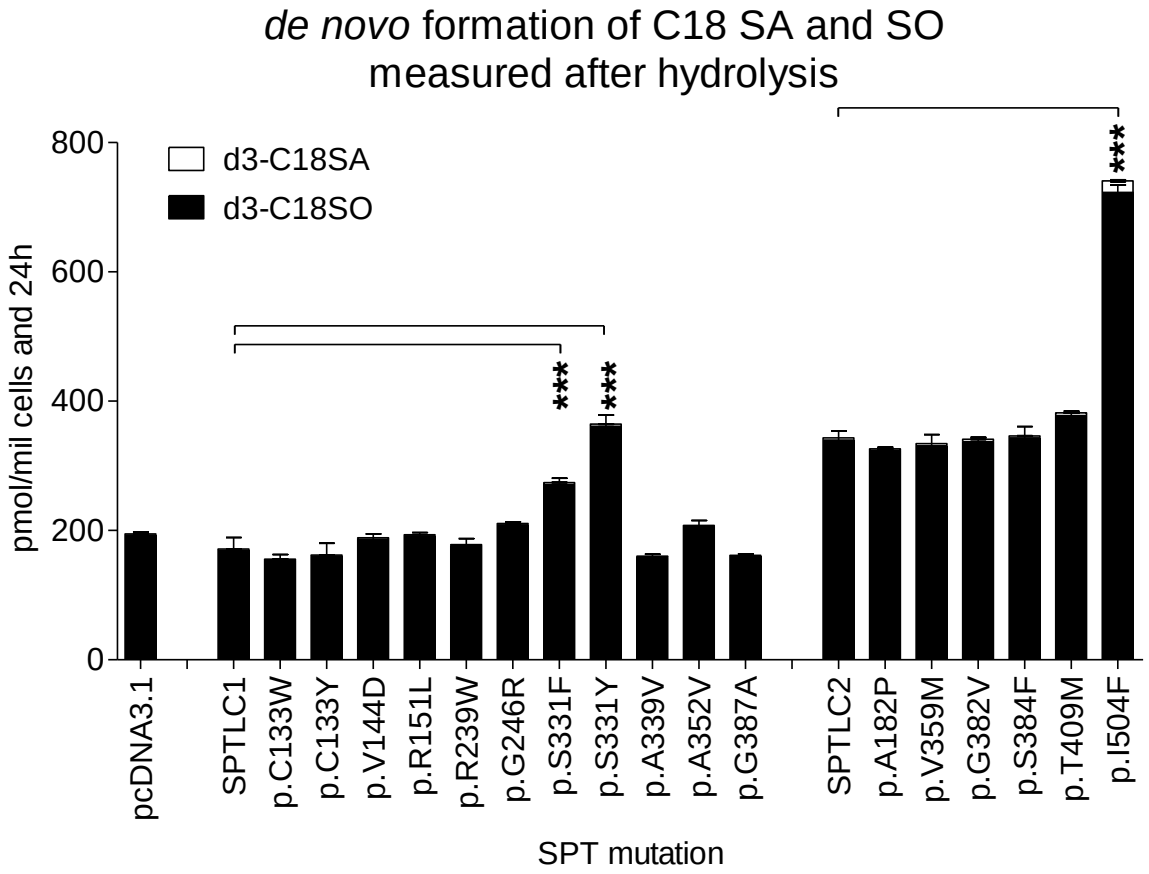


Figure 5-4: Canonical SPT activity was measured in HEK293 cells overexpressing HSAN1 mutations or the wild type SPTLC1 and SPTLC2 subunits.
The cells were incubated for 24 hours in medium containing the stable isotope labelled amino acids d3-N_{15}1-L-serine and d4-L-alanine (1 and 2 mM respectively). The *de novo* formed, labeled sphingoid bases were quantified by LC-MS and corrected for isotopic distribution. Significance of differences was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001.

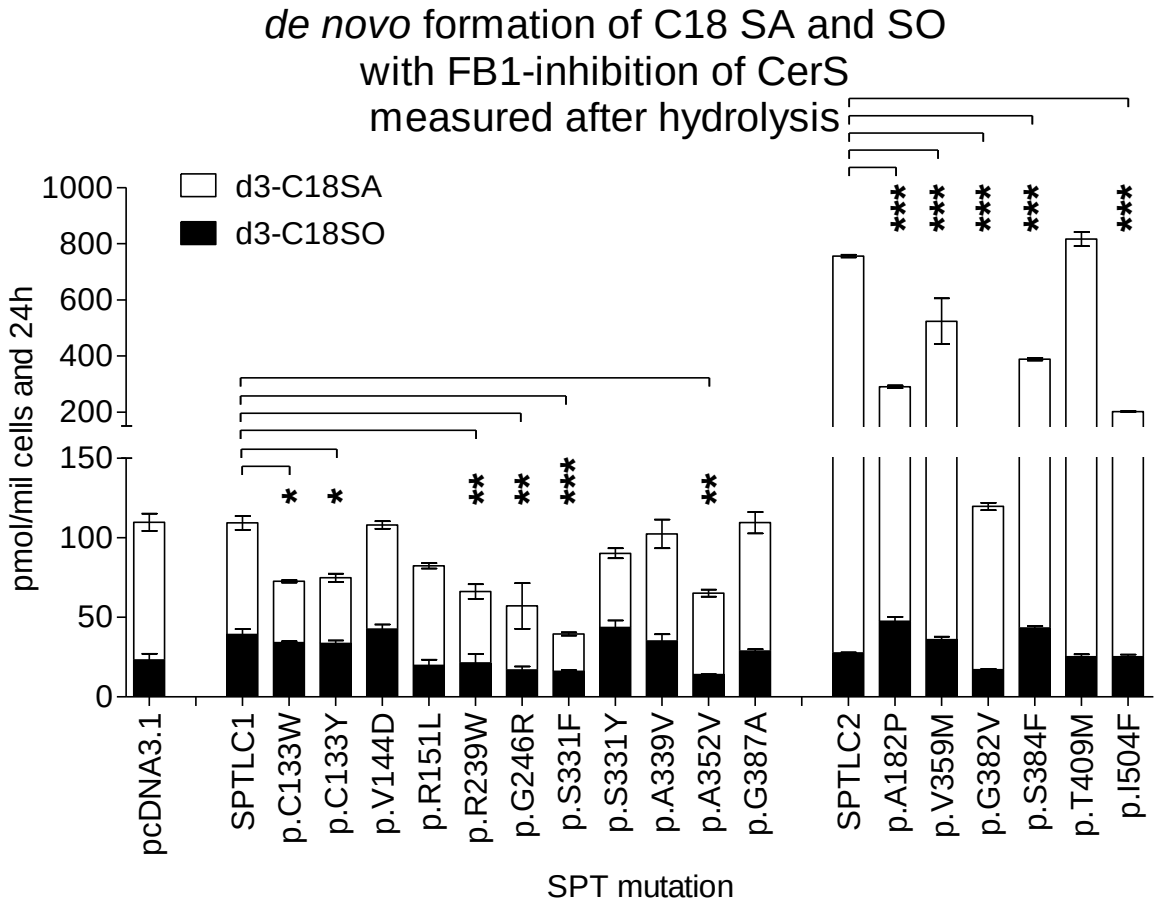


Figure 5-5: Cellular canonical SPT activity in HEK293 cells in presence of the Cers inhibitor Fumonisin B1. Cells were incubated for 24 hours in medium containing the stable isotope labelled amino acids d3-N_{15}1-L-serine and d4-L-alanine (1 and 2 mM respectively). Activity of ceramides synthase was blocked within this incubation period by simultaneous addition of 30µM of the mycotoxin fumonisin B1. The *de novo* formed, labeled sphingoid bases were quantified again by LC-MS and corrected for isotopic distribution. The reduction of SPTs canonical activity by the different mutations was compared to the respective wild types Significance of differences was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001, **p < 0.01 and *p < 0.05.

5.4.3 Effects of the different mutations on the formation of 1-deoxysphingolipids

Besides the effects on the canonical pathway leading to formation of normal C18 sphingolipids, we were especially interested in the effects of the different mutations on the formation of the atypical and neurotoxic 1-deoxysphingolipids (1-deoxySL).

In a first approach we measured the incorporation of d4-L-alanine into 1-deoxysphingolipids within 24 hours in the absence of FB1 (see Figure 5-6).

The basal levels of 1-deoxySL generated by wild type SPTLC1 and 2 were not increased in comparison to the empty vector control, suggesting that an overexpression of SPTLC1 or SPTLC2 alone does not cause an increased activity with L-alanine.

In contrast to the wild type subunits the expression of several but not all mutants caused a significant increase of 1-deoxySL formation. The SPTLC1 mutations p.C133W, p.C133Y, p.S331F and p.S331Y showed a significant higher activity with L-alanine resulting in increased 1-deoxySL *de novo* synthesis. The same was seen for the SPTLC2 mutations p.A182P, p.G382V, p.S384F and p.I504F. Cells expressing the SPTLC1 variants p.V144D, p.G246R, p.A339Vand p.A352V and SPTLC2 p.V359M and p.T409M showed no significantly altered activity with L-Alanine compared to SPTLC1 wt.

In the presence of FB1 (see Figure 5-7) 1-deoxySA formation followed the same pattern as seen in the untreated cells before. Significantly elevated 1-deoxySL levels were again measured in cells expressing the SPTLC1-mutations p.C133W, p.C133Y, p.S331F, p.S331Y and the SPTLC2-mutations p.A182P, p.G382V, p.S384F and p.I504F. In contrast SPTLC1 p.V144D, p.G246R, p.A339Vand p.A352V and SPTLC2 p.V359M and p.T409M were not associated with increased 1-deoxySL formation. However, although the general pattern for the mutants did not change in the presence or absence of FB1, the total amount of incorporated d4-L-alanine in the presence of FB1 was higher for several of the analyzed mutants. The 1-deoxySL formation in the p.C133W and p.C133Y mutations was stimulated by FB1 (4.5- and 2.3-fold in comparison to the previous assay (see Figure 5-6) whereas this effect was less pronounced for the p.S331F and p.S331Y (1.3- and 1.8-fold respectively). Similar observations were made for the SPTLC2 mutations. For the p.A182P variant we observed a more than 10-fold increased incorporation of d4ala in the presence of FB1 while the p.I405F mutant-expressing cells incorporated about 5-fold more d4-L-alanine.

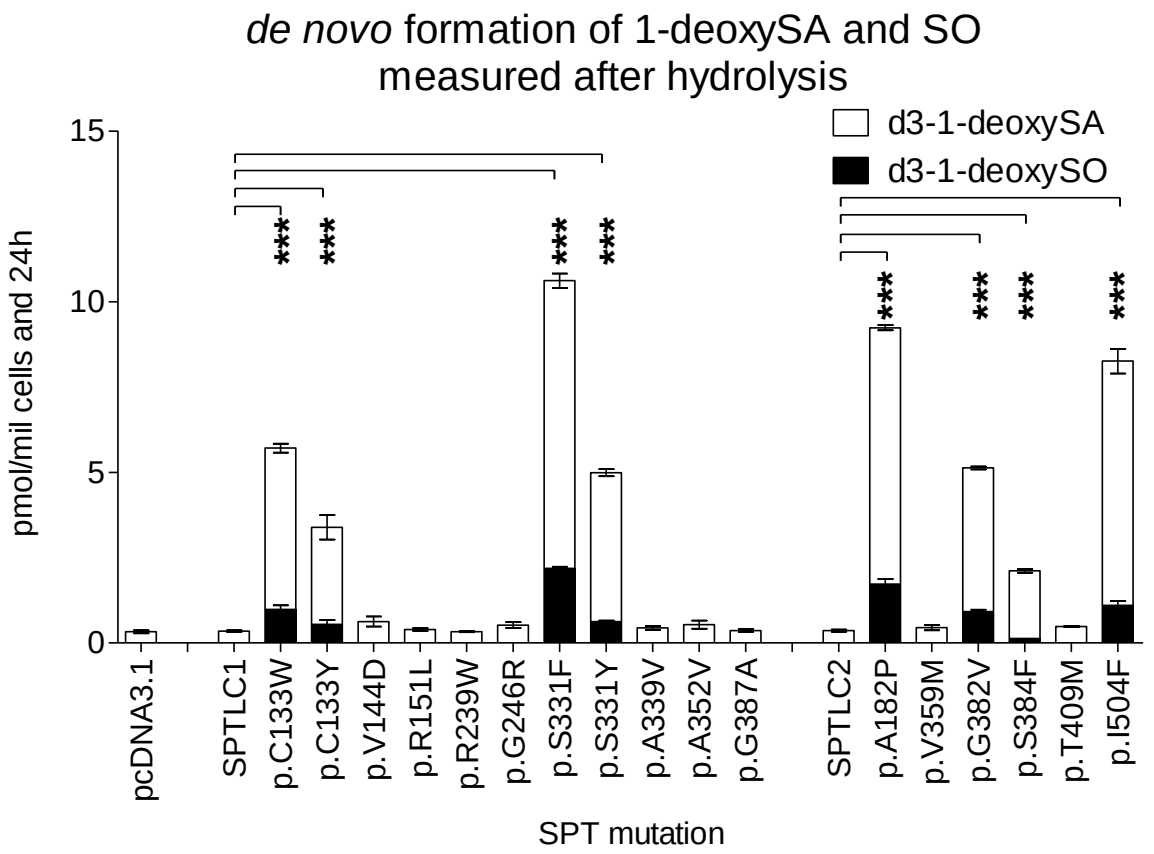


Figure 5-6 Intracellular formation of atypical 1-deoxySL by SPT under physiological conditions. Overexpression of SPTLC2 did not increase the generation of the atypical 1-deoxysphingolipids. Cells expressing the SPTLC1-mutations p.C133W,p. C133Y, p.S331F and p.S331Y and the SPTLC2-mutations p.A182P, p.G382V, p.S382V and I504F respectively possessed a significantly elevated activity with Alanine (9-30 folds higher for the SPTLC1 mutations and 6-25 folds for the mutations in SPTLC2). Significance of differences was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001.

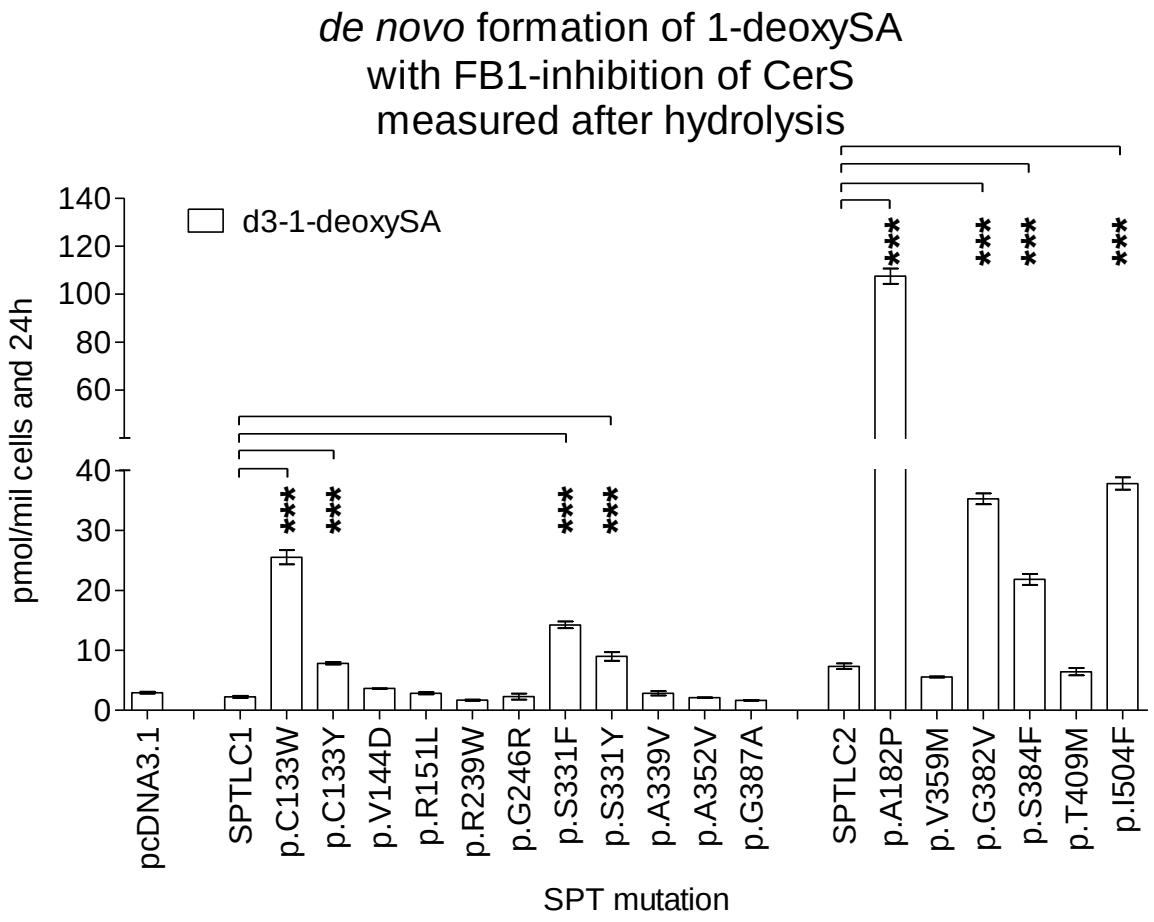


Figure 5-7: Cellular SPT activity with L-alanine in HEK293 cells overexpressing HSAN1 mutant or WT Subunits of SPT (SPTLC1 and SPTLC2). Generation of the atypical 1-deoxysphinganine was significantly increased for the SPTLC1-mutants C133W, C133Y, S331F and S331Y as well as for the SPTLC2-mutants A182P, G382V, S382V and I504F in direct comparison to their respective WT subunits with P<0.001. Significance of differences was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001, **p < 0.01 and *p < 0.05.

5.4.4 Effects of HSAN1 mutations on the generation of minor sphingolipid species

Besides palmitoyl-CoA with its 16 carbon atoms, SPT can metabolize other acyl-CoAs with carbon chain lengths in the range of C14-C18. This results in the formation of sphingoid bases with different chain lengths. For Example condensation of stearoyl-CoA and L-serine causes the formation of C20 sphingoid bases. Sphingoid bases with carbon chain lengths other than C18 are usually formed in significantly lower amounts and referred to as minor sphingoid bases. The formation of C16 sphingoid base backbones depends on the presence of the SPTLC3 subunit in the SPT complex while C20 sphingoid bases can also be formed in the absence of SPTLC3. Comparing the HSAN1 mutants for their ability to form minor sphingoid bases, we observed a significantly increased formation of C20 sphingoid bases for the SPTLC1 p.S331F, p.S331Y and SPTLC2 p.I504F mutants, which was not seen for any of the other mutants (see Figure 5-8).

This finding was further confirmed in plasma from HSAN1 patients with the p.S331F and p.S331Y mutation which also showed significantly increased C20 SL levels (see chapters 2 and 3 of this thesis).

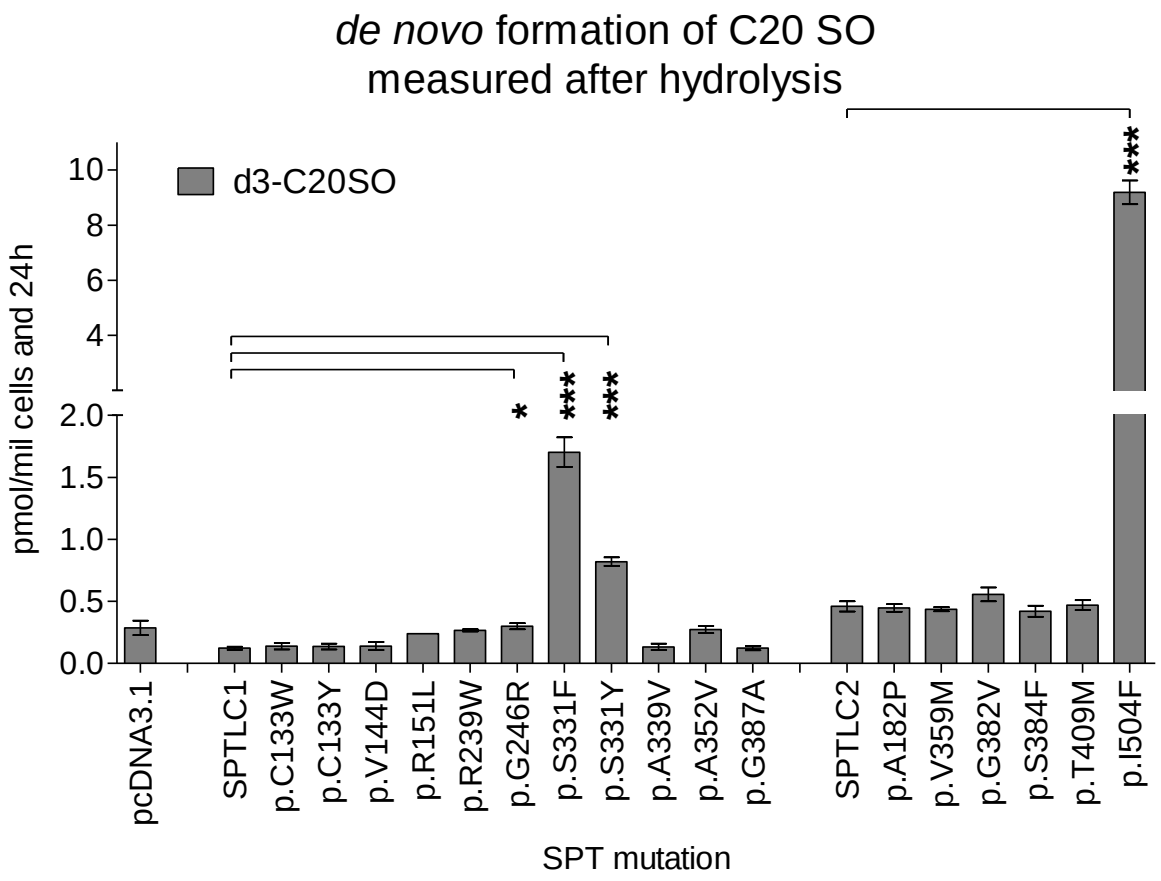


Figure 5-8 Alteration of long chain sphingoid base production by mutations in SPT. Cells were incubated for 24 hours with d3-N_{15}1-L-serine and d4-L-alanine (1 and 2 mM respectively) in absence of FB1. The *de novo* formed, labeled sphingoid bases were measured by LC-MS, normalized to 200pmol d7-SA and corrected for isotopic distribution. Significance of changes compared to the respective wild types was tested by 1-way ANOVA followed by Dunnetts multi comparison test and is indicated by stars with * and * indicating a significant difference with p<0.05 and p<0.001 respectively. Values for most of the measured cell lines were close to or below the detection limit of ~1pmol/mil cells. The mutants p.S331F, p.S331Y and p.I504F were the only ones to produce easily measurable concentrations of d3-C20SO.**

5.4.5 Comparative analysis of SPT mutations based on their biochemical properties

Clustering of the mutations reveals genotype phenotype correlation between the different groups.

To analyze a potential genotype-phenotype relationship of the mutants we calculated the ratios between mutant and wild type to get fold changes instead of absolute values for the measured sphingolipid species. The correlation between the ratios of 1-deoxySL and C20 SL showed the strongest discrimination power resulting in the formation of three distinct clusters of mutations (see Figure 5-9).

The 1st group comprises all mutations, which do not or only minimally influence the activity with serine or alanine. These SPT mutants did not form considerable amounts of 1-deoxy nor C20-sphingolipids and behaved similar to the wild type subunit.

The 2nd cluster included mutations that showed an increased formation of 1-deoxysphingolipids. This group covered the two well described HSAN1 mutations in SPTLC1 (p.C133W and p.C133Y) and several mutations in SPTLC2 (p.A182P, p. G382V and p.S384F). These mutations are associated with an average age of disease onset between 2nd and 3rd decade and moderate motor impairment. The SPTLC2 mutation p.A182P is special and obviously marks the outer edge of this “classical” group, as it is associated with the highest increase in 1-deoxySL formation. The p.A182P mutation was identified in patients with a more severe phenotype with early onset and pronounced motor involvement. However, the absence of elevated C20-sphingoid bases and the lack of atypical symptoms in the patients nevertheless suggest that p.A182P is associated with a severe but typical HSAN1C phenotype. The mutations C133Y and C133W are the most frequent mutations for HSAN1 and were found in several large kinships in Europe, the US and Australia. The two SPTLC2 mutations p.G382V and p.S384F were reported to be associated with a regular HSAN1C phenotype.

The 3rd group consists of three mutations (SPTLC1 p.S331F, p.S331Y and SPTLC2 p.I504F) which affect the amino acid but also the acyl-CoA preference of SPT, causing both, an increased formation of 1-deoxy- and C20 sphingoid bases. The increased formation of C20-based sphingolipids is unique amongst this group and represents a clear hallmark. These three mutations also appear less susceptible to the activity inducing effects of FB1 on 1-deoxySL-formation and showed the strongest reduction of canonical activity in the presence of this CerS inhibitor. All mutations combined in this group are associated with a severe phenotype characterized by early or even congenital onset, growth retardation, autonomic impairments (like temperature regulation by sweating), juvenile cataracts and involvement of the respiratory system.

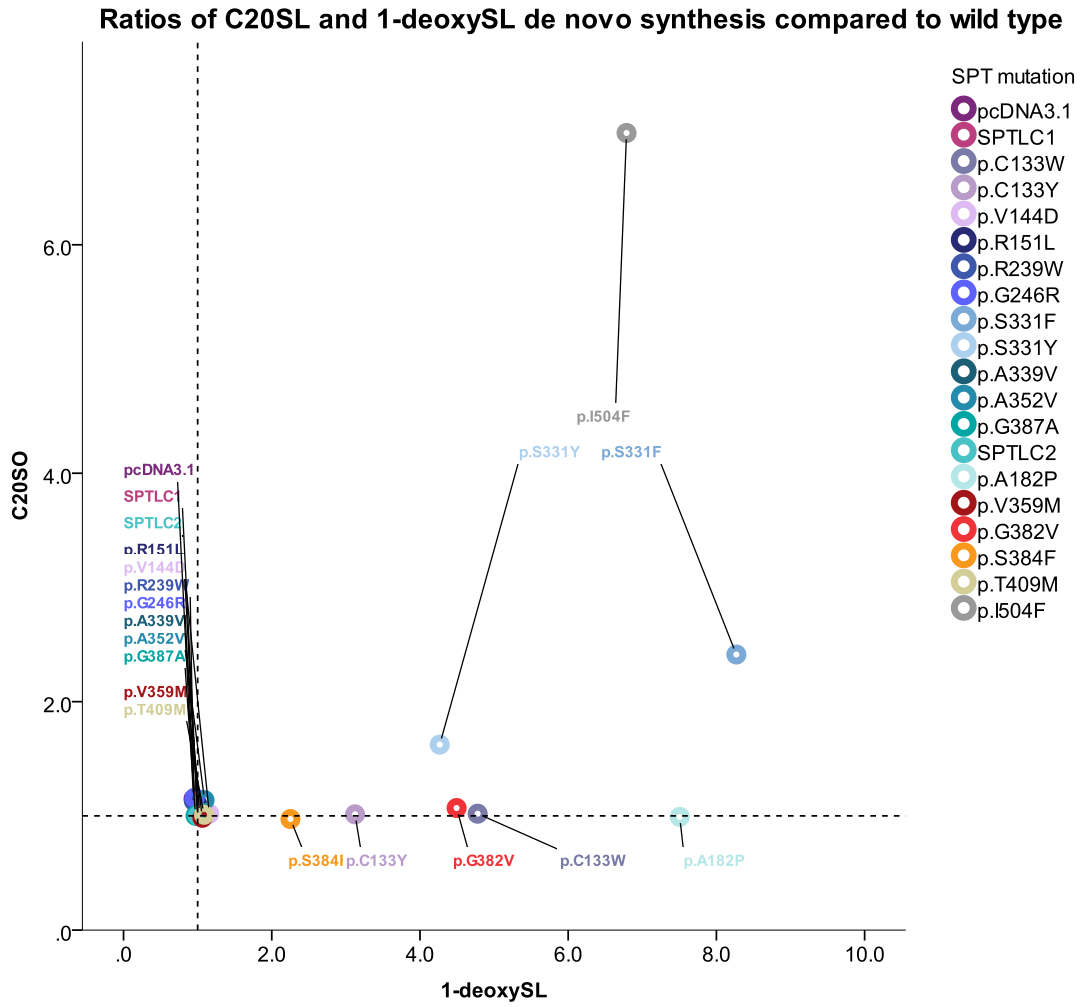


Figure 5-9 Grouping of different HSAN1 mutations based on intracellular formation of C20-based sphingolipids and 1-deoxysphingolipids as measured in standard conditions. The levels of *de novo* formed C20 and 1-deoxysphingolipids were isotopic corrected and normalized to the internal standard and 1mil cells. Final concentrations were divided by the matching wild type expressing controls and are presented as fold change.

We also performed a principal component analysis (PCA), including more sphingoid bases, using the multivariate data analysis tool SIMCA-P+. Besides the two ratios of C20 SL and 1-deoxySL in comparison to wild type (see Figure 5-9 and Figure 5-12), we added the ratios of four other *de novo* formed sphingoid bases (C17SO, Sphingadiene, C18SA and C18SO) and divided the 1-deoxySL into the two individual sub species 1-deoxySA and 1-deoxySO.

The multivariate analysis of these seven sphingoid base ratios resulted in a comparable clustering as shown above (compare Figure 5-9 and Figure 5-10). The graphical presentation allowed the grouping of the 20 mutants into three clusters again (Clusters 1, 2 and 3 are highlighted in blue, orange and red respectively, see Figure 5-10).

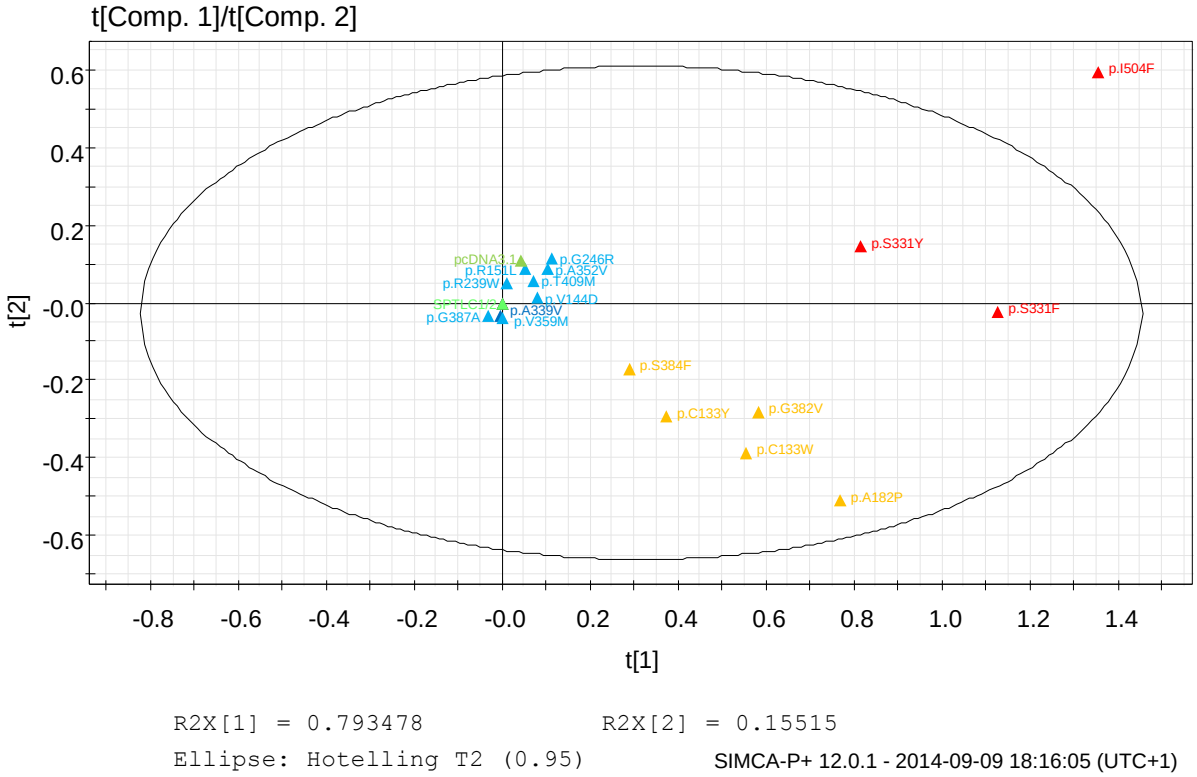


Figure 5-10 Score scatter plot of the multivariate analysis of all mutants. Graphical proximity reflects the degree of correlation of the underlying parameters

The assigned discriminatory weight for the suggested model identified C20SO on the one and the two 1-deoxySL species on the other side as the strongest discriminators for the clustering of the mutants (see Figure 5-11).

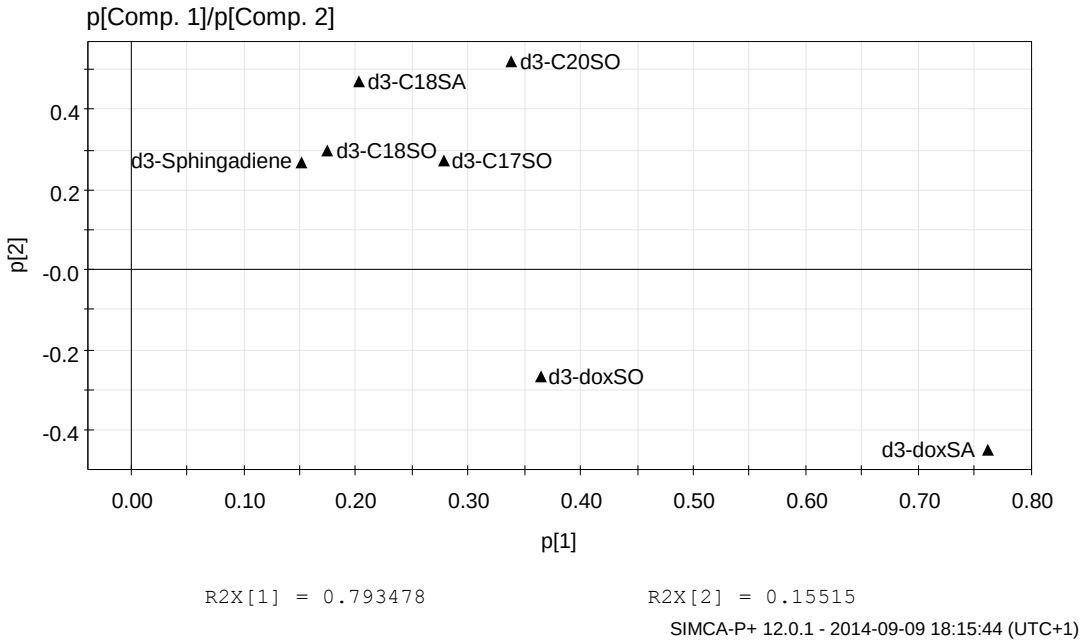


Figure 5-11 Loading scatter plot of the conducted PCA. Distance and direction from the origin of the axes equals the assigned weight and importance of the individual sphingolipid species for the grouping of the mutants.

To challenge these *in vitro* observations, we furthermore correlated the ratios of plasma concentrations of the strongest discriminatory species (1-deoxySL and C20 SL) from HSAN1 patients with different mutations in SPT. Most of the plasma data presented in Figure 5-12 results from old patient samples from previous and already published studies. The plasma of these patients had been stored at -80°C and was extracted and measured again for the comparison. For the new patients (SPTLC1 p.S331F and p.A339V), patient and control plasma was provided by the neurologists in charge, Ekkehard Wilichowski and Joachim Weis. In this analysis we could only compare seven out of 17 mutations as plasma samples for the other mutations were not available.

Figure 5-12 presents a similar pattern as the cell culture model and the extended PCA analysis (see Figure 5-9 and Figure 5-10). Mirroring the *in vitro* results, the SPTLC1 p.R151L mutation did neither increase plasma concentrations of C20 SL nor 1-deoxySL. Patients with the three classical mutations (SPTLC1 p.C133W, p.C133Y and p.V144D) and also the newly found p.A339V mutation presented elevated plasma levels of 1-deoxySL (range: 2-22-folds increased compared to healthy controls) while the C20 SL levels remained unchanged (less than 2-folds increased). Plasma samples from totally three patients with mutation at p.S331 (patients were measured repeatedly over several months/years, see chapter 3 of this thesis) had clearly elevated concentrations of 1-deoxySL but also of C20 SL which again confirmed the results from the cell culture model.

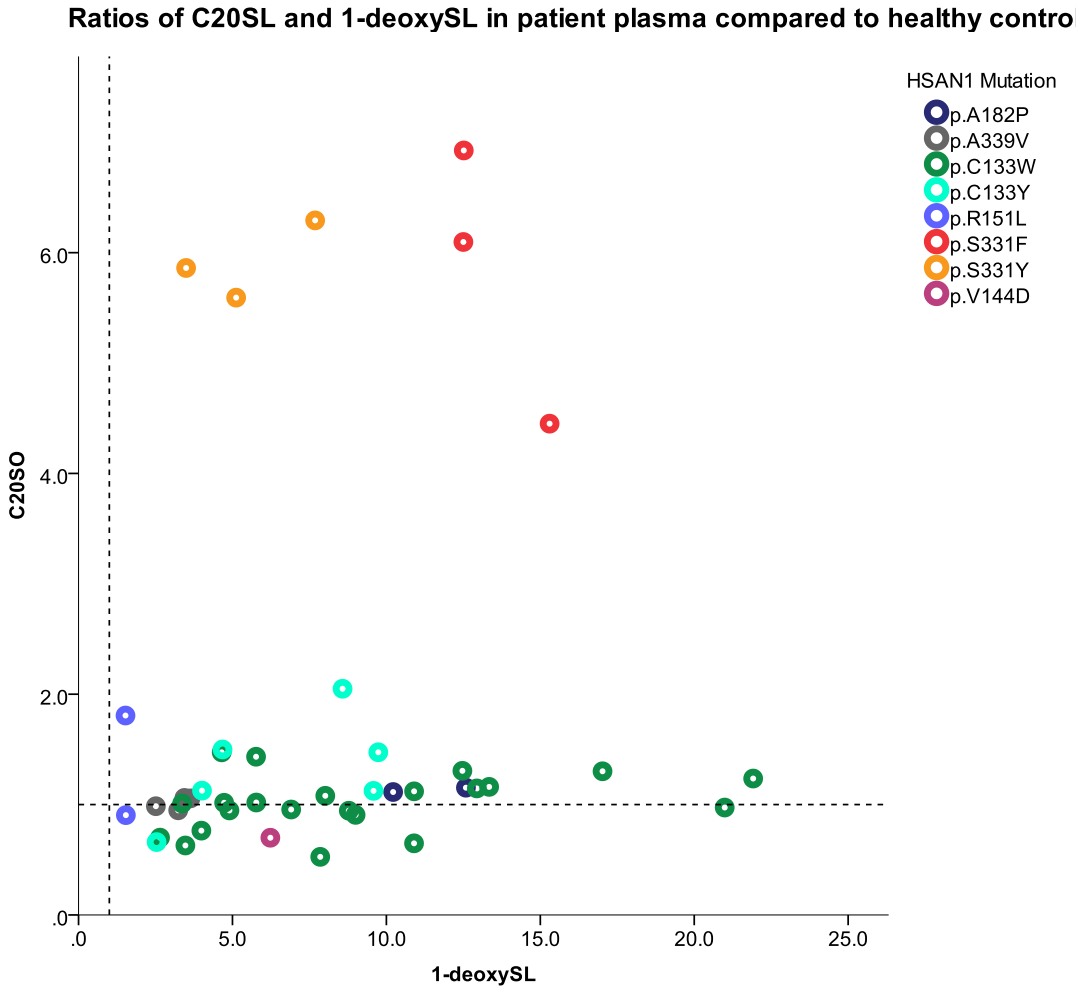


Figure 5-12 Grouping of different HSAN1 mutations based on lipidconcentrations measured in plasma of affected patients. The plasma levels of C20 and 1-deoxysphingolipids were normalized to the internal standard and 100µl extracted samplevolume. The resulting plasmaconcentrations in the patient samples were divided by the average concentration of healthy controls measured in the same run and are shown as fold change compared to this control.

An increased canonical activity together with altered preferences of SPT for its amino acids and acyl-CoA substrates was observed exclusively in the three mutations. Increased levels of 1-deoxySL and C20 SL were reproducibly detected in plasma of patients carrying these mutations. All mutations affecting the residues S331 in SPTLC1 or I504 in SPTLC2 cause an extraordinary severe phenotype characterized by early onset, growth and in one case even mental retardation, development of cataracts and respiratory complications (for an overview please see Table 6). The symptoms observed in this group of patients were distinct from the other HSAN1 patients. Correlation of phenotype and genotype is obvious for these mutations.

Mutation	origin/ inheritance	AAO (years)	type of polyneuropathy	weakness	ulcers/ amputation	respiratory problems	cataracts (age)	other symptoms	Reference (Author, year)
SPTLC1 p.C133W	Australian English/NM	65	NM	Yes in 4/38	Yes	NM	NM	NS	Dawkins *et al*, 2001
	Canadian/NM	20-40	NM	Yes in 6/7	Yes	NM	NM	NS	Bejaoui *et al*, 2001
	Chinese/AD	20's	sensory motor	No	Yes	NM	NM	NS	Bi *et al*, 2007
	Canadian/AD	12, 60's	sensory	peroneal atrophy	Yes	NM	NM	NS	Klein *et al*, 2005
	English/AD and IC	12 – 70, Avg=29	sensory motor	Yes	Yes	NM	NM	NS	Houlden *et al*, 2006
SPTLC1 p.C133Y	German/NM	NM	NM	NM	NM	NM	NM	NS	Bejaoui *et al*, 2001
	Australian German/NM	NM	NM	NM	NM	NM	NM	NS	Dawkins *et al*, 2001
	Portuguese/AD	20's, 10	sensory motor	No	No	NM	No	Foot pain, dry skin	Geraldes *et al*, 2004
SPTLC1	German/AD	50	None	No	NM	NM	NM	NS	Rautenstraus

p.C133R									s *et al*, 2009
SPTLC1 p.V144D	Australian German/NM	NM	NM	NM	NM	NM	NM	NS	Dawkins *et al*, 2001
SPTLC1 p.A310G	English/IC	50's	sensory	No	Yes	NM	NM	NS	Davidson *et al*, 2012
SPTLC1 p.S331F	German/IC	Early childhood	sensory motor	Yes	Yes	NM	Yes (9)	sweating disturbances and anhydrosis, joint contractures, fractures	Huehne *et al*, 2008
	French (Gypsy)/IC	congenital	sensory motor	Yes	Yes	Yes	Yes	joint hyperlaxity, severe growth and mental retardation, microcephaly, hypotonia, vocal cord paralysis, gastro-oesophagial reflux	Rotthier *et al*, 2009
	Korean/IC	5	sensory motor	Yes walker (27)a	Yes	Yes	Yes (10)	Hoarseness, tremor, scoliosis	Suh *et al*. 2014
SPTLC1 p.S331Y	Austria/IC	4	sensory motor	Yes wheelchair (14)a	Yes	Yes	Yes (13)	Tremor, fasciculation, joint hypermobility, pes cavus	Auer-Grumbach *et al,* 2013

SPTLC1 p.A352V	Austrian/IC	16	sensory motor	, distal LL, peroneal atrophy	No	NM	NM	Mild pes cavus, lancinating pains	Rotthier *et al*, 20009
SPTLC2 p.V359M	Austrian/IC	52 yrs	axonal/intermediate sensory motor	NM	Yes /great R toe	NM	NM	ulceration and amputation of great R toe	Rotthier *et al*, 2010
SPTLC2 p.G382V	German/AD	37yrs	axonal/intermediate sensory motor	Yes UL and LL	No	NM	NM	dysesthesia and sensory loss distal UL and LL	Rotthier *et al*, 2010
	Austrian/AD	38 yrs, mother asymptomatic	axonal sensory motor	Yes LL	No	NM	NM	sensory loss in feet	Rotthier *et al*, 2010
SPTLC2 p.I504F	Czech/IC (*de novo*)	5 yrs	intermediate sensory motor	Yes (LL)	Yes (LL)	NM	NM	sweating disturbances and anhydrosis, gait difficulties, foot deformities	Rotthier *et al*, 2010

Table 6 Overview of clinical features of HSAN patients with SPTLC1 mutation (combined and adapted from (21, 25, 35)) AAO, age at onset; PN, polyneuropathy; IC, isolated case; AD, autosomal dominant; SM, sensory motor polyneuropathy; NM, not mentioned; LL, lower limbs; UL, upper limbs; NS, not specified

5.4.6 Effect of high doses of amino acid substrates on 1-deoxySL formation and general activity of HSAN1 mutants

The formation of 1-deoxySL strongly depends on the type and concentration of the available amino acid substrates. We reported earlier that supplementation with high concentrations of L-serine efficiently lowers the 1-deoxySL formation in p.C133W cells (see Figure 5-13) and even the 1-deoxysphingolipid levels measured in plasma of transgenic mice expressing the p.C133W mutation could be decreased by oral administration of L-serine. In reverse, the availability of higher amounts of L-alanine increased the formation of 1-deoxySL in the cells (see Figure 5-13) and was also shown to aggravate the phenotype of HSAN1 mice (41).

We measured the 1-deoxySL formation of cells expressing the SPTLC1 wild type and the p.C133W mutation at three different amino acid concentrations (see Figure 5-13). As described above, p.C133W expressing cells formed increased concentrations of 1-deoxySL in our standard conditions (1mM d3-L-serine and 2mM d4-L-Alanine) compared to the wild type. In the presence of 10mM d3-L-serine this increased 1-deoxySL formation was reduced and did not significantly deviate from SPTLC1 wild type expressing cells. Supplementation with 10mM d4-L-alanine instead caused a significantly elevated 1-deoxySL concentration in the p.C133W expressing cells which were 4-folds higher than under standard conditions. The increase upon alanine stimulation was significant in comparison to the SPTLC1 wild type cells, which did not form more 1-deoxySLs in response to higher L-alanine concentrations.

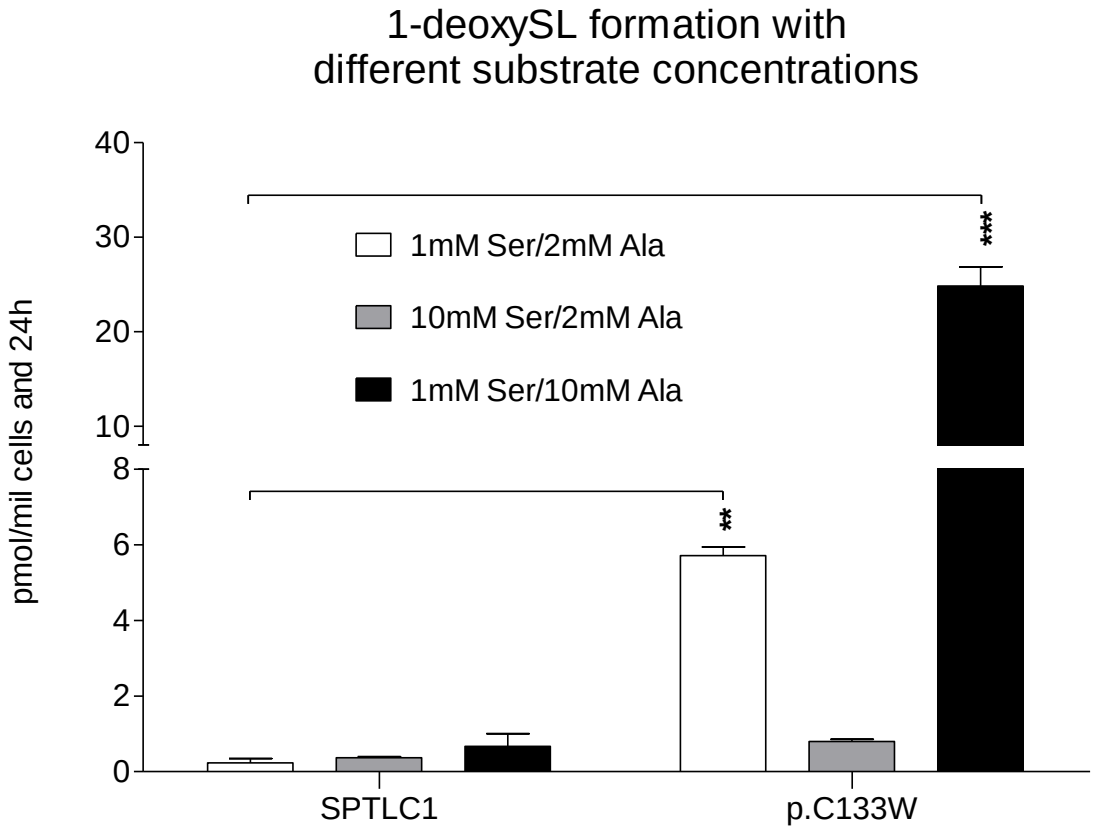


Figure 5-13 *De novo formation of* 1-deoxySL with different substrate concentrations. HEK 293 cells expressing the SPTLC1 mutation p.C133W were incubated for 24 hours in amino acid free medium withdifferent ratios of of d3-L-serine and d4-L-alanine (1mM Ser/2mM Ala, 10mM Ser/2mM Ala and 1mM Ser/10mM Ala). The formed 1-deoxySL were hydrolyzed, measured by LC-MS and normalized to cell number and 200pmol of internal standards. 1-way ANOVA was performed to detect differences between the obtained values which were then analyzed for significance by comparison to the SPTLC1 expressing cells in standard medium, using Dunnett's multi comparison test. Significance of differences is shown as *, with ***p < 0.001 and **p < 0.01.

Based on previous results we were interested to see whether the 1-deoxySL lowering effect of L-serine was also effective for other HSAN1 mutations.

Like before, Hek293 cells transfected with the different mutants were treated with either 10mM d3-L-serine or 10 mM d4-L-alanine (on a background of 2mM d4-L-alanine or 1mM d3-L-serine respectively).

Figure 5-14 showed the effects of high dose amino acid stimulation on 1-deoxySL formation in wild type or mutant-expressing cell lines. Supplementation with high dose L-serine resulted in a significant reduction of the 1-deoxySL in the mutants SPTLC2 p.A182P and SPTLC2 p.I504F, which were already shown to form elevated levels of 1-deoxySL under standard conditions. Increased L-serine levels reduced 1-deoxySL formation also in the SPTLC1 p.S331F expressing cells but to a lower extent (>1pmol/mil cells and 24h) compared to other mutants. The low basal 1-deoxySL levels in SPTLC1, SPTLC2 and SPTLC1 p.A339V expressing cells could not be lowered further.

Higher concentrations of L-alanine caused a significant increase in 1-deoxySL formation in all mutant-expressing cells and also in SPTLC2 wt expressing cells but had no effect on SPTLC1 wild type expressing cells. At 10mM L-alanine the p.A339V mutation showed a significantly increased 1-deoxySL formation also.

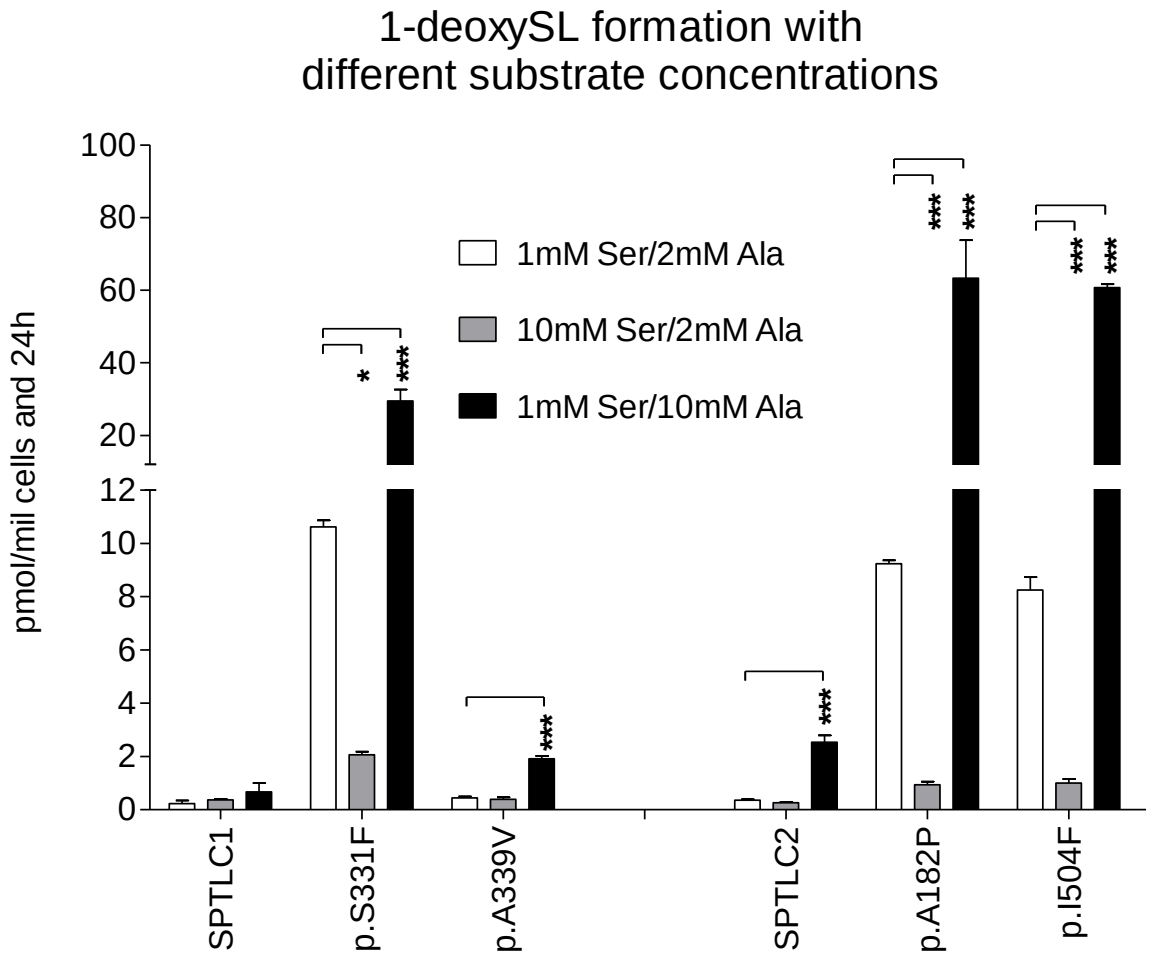


Figure 5-14 1-deoxySL formation with different substrates. HEK 293 cells expressing the SPTLC1 mutation p.C133W were incubated for 24 hours in amino acid free medium with different concentrations of d3-L-serine and d4-L-alanine. The formed 1-deoxySL were processed and analyzed as before. Significance of mutation-specific differences between the three treatments was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001 and *p < 0.05.

Accordingly we expected that an increased availability of L-serine would stimulate the formation of sphingolipids in general as measured by the concentrations of the canonical sphingoid bases (SA and SO).

Compared to the standard conditions, the supplementation with 10mM of d3-L-serine resulted in an increase of canonical activity of SPT in all cell lines (see Figure 5-15). Incorporation of d3-L-serine was moderately increased in SPTLC1 (1.4-fold) and SPTLC2 (1.9-folds) transfected cells. The canonical activity of the mutants SPTLC1 p.C133W, p.A339V and SPTLC2 p.A182P was not different from the respective wild type controls and was similarly increased in the presence of 10mM d3-L-serine. However, cells expressing SPTLC1 p.S331F and SPTLC2 p.I504F presented both with a 3- and 2-fold increased activity in the presence of 10mM d3-L-serine. For the p.S331F mutant we observed a significantly reduced incorporation of L-serine upon stimulation with high doses of L-alanine. The other mutants and wild type expressing cells showed no effect on the formation of the canonical SPT products at high alanine concentrations.

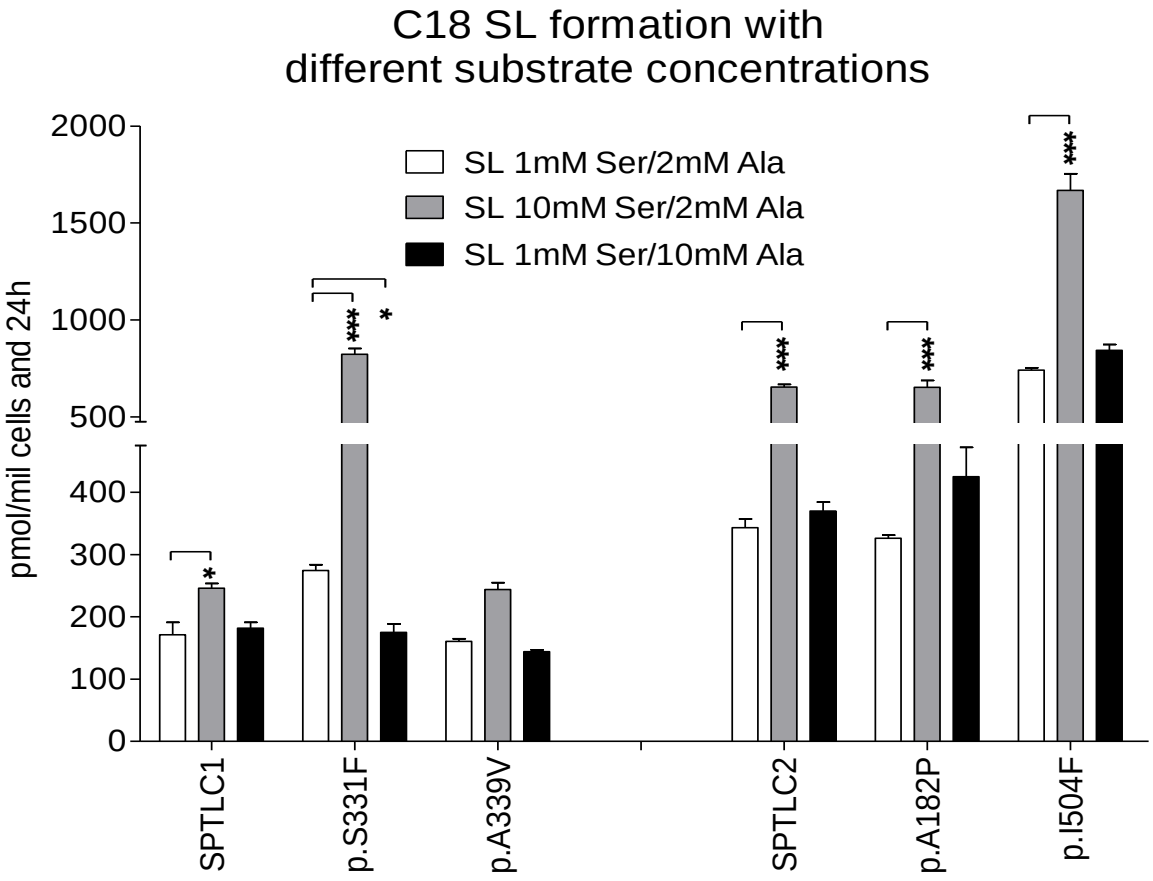


Figure 5-15 Canonical SPT activity with different substrates
Cells were cultured in normal assaymedium or medium supplemented with 10mM d3-L-serine or d4-L-alanine respectively. Significance of differences was tested by 1-way ANOVA followed by Dunnett's multi comparison correction and is shown as *, with ***p < 0.001 and *p < 0.05.

5.5 Discussion:

With this study, we provide the first comprehensive biochemical analysis covering the sphingolipid profiles of 17 different mutations of serine palmitoyltransferase subunit 1 and 2. The fact that also the lower expressed mutants p.A339V and p.A352V were able to keep pace with the other SPTLC1 mutants, shows once more that the expression levels of SPTLC1 do not have a major impact on SPT´s canonical activity (see Figure 5-2 A). This cannot be expected from cells overexpressing the SPTLC2 subunit. The weaker signal for the p.G382V, p.S384F and p.T409M expressing cells (see Figure 5-2 B) rather results from a lower total protein concentration in these samples. This is also supported by the fact that as for the SPTLC1 mutants, all SPTLC2 mutants, apart from the overactive p.I504F, showed identical canonical activities and the same levels of C18 based SL as SPTLC2 wt expressing cells. All together we assume that the differences in expression of our constructs were minor and did not significantly influence our later measurements.

Metabolic labeling with deuterated substrates allowed us to determine the activities of the different SPT variants in the cellular context using intact and living cells, in more physiological conditions and without any interference with the *de novo* synthesis pathway. For our experiments, the cells were cultured in monolayers and we assume, they stayed alive and intact during the treatment, the substrate concentrations were close to physiological conditions and far from saturating conditions as it is the case for the *in vitro* enzyme assays and we assume that neither localization nor interaction with potential intracellular regulatory proteins were altered.

Metabolic labeling of sphingolipids was possible and performed before, e.g. using radiolabelled substrates L-[^{3}H] or L-[U-^{14}C]serine substrates (10, 42, 43). However, the clear advantages of the metabolic labeling approach, as we conducted it are the close proximity to the complex situations in whole organs and even organisms and the unproblematic, quantitative and sensitive analysis of the extracted lipids by LC-MS compared to TLC and autoradiography or scintillation counting..

Interestingly, despite earlier *in-vitro* results we did not see any reduced canonical activity of the mutants (see Figure 5-4) in the isotope labelling assay suggesting that intracellular SPT activity in our cell culture model even in cells overexpressing the mutated subunits was still sufficient to maintain intracellular sphingolipid formation. Even challenging the mutants with high doses of 10mM L-serine was not sufficient to display differences in canonical activity in the mutant-expressing cells (see Figure 5-15). These two observations confirm and support our earlier report, that HSAN1 is caused by a gain of function resulting in the formation of 1-deoxysphingolipids rather than by the loss of canonical SPT activity (28). This is further

supported by Dedov and colleagues who showed that even at reduced canonical SPT activity as measured by the L-[^{3}H]serine *in vitro* assay total intracellular sphingolipids levels were not altered (44).

Nevertheless our observations partially conflict with other studies, reporting mutations in SPT to cause reduced canonical activity (37–39). However, these results were mostly obtained using *in vitro* conditions as described by Merill (42, 43) or after the accumulation of free sphingoid bases upon addition of the CerS inhibtor Fumonisin B1 as described by Wang and colleagues (45, 46).

These two approaches are distinct from ours, which uses intact cells and avoids interference with the downstream metabolism of sphingoid bases.

There is increasing evidence that *de novo* SL synthesis is a highly regulated process. Several smaller interacting proteins were described to influence SPT's activity and substrate preferences.

In 2010 the yeast proteins Orm1 and Orm2 were reported to modulate sphingolipid homeostasis by down-regulation of SPT activity via negative feedback mechanisms (47). Affinity tagged Orm1 and Orm2 proteins co eluted with Lcb1, Lcb2, Sac1 and Tsc3, suggesting the presence of a higher order complex of SPT the so called SPOTS complex (serine palmitoyltransferase, Orm1/2, Tsc3 and Sac1). It is believed that within this complex, dephosphorylated Orm proteins bind the Lcb1/2/Tsc3 heteromer of SPT and inhibit its function. Sac1 binds independently of the Orms to the complex and seems to exert an inhibitory effect on the functional SPT complex as well (47, 48). Upon intracellular ceramide shortage e.g. by inhibition of SPT with the mycotoxin Myriocin, Orm proteins get gradually phosphorylated by the Ypk1 kinase and dissociate from the SPT complex together with Sac1 to finally relieve SPT from its inhibition (47, 49). Once the sphingolipid levels are restored, the Orm proteins are dephosphorylated again, reestablishing the inhibition of SPT (47). Allthough these results were obtained in yeast, the mechanisms might be similar in higher eukaryotes .The involved proteins are highly conserved and three orthologous proteins of Orm1 and 2 were found in human and named ORMDL1-3 (50). In 2009 two orthologous of the yeast protein Tsc3 (small subunit SPT a and b; ssSPTa and ssSPTb) were identified in the human genome and characterized (51). The authors showed that co expression of the small subunits together with SPT in yeast or mammalian cells increased SPT's activity 50-100-fold. Apart from this induction of SPT activity the ssSPTs also affected the acyl-CoA preference of the formed SPT complex. Co expression of the human SPT subunits SPTLC1 together with SPTLC2 and ssSPTa in yeast SPT knockout strains resulted in formation of the common C18 based sphinoid backbones, while cells expressing SPTLC1, SPTLC2 and

ssSPTb preferred stearoyl-CoA and formed increased amounts of C20-based sphingolipids (51).

Besides three exceptions the canonical activities of the SPTLC1 and SPTLC2 mutants were all constant within a narrow range. None of the mutations caused a reduced canonical activity. In contrast three mutants (SPTLC1 p.S331F, p.S331Y and SPTLC2 p.I504F) had a significantly increased activity with L-serine. We assume that SPT in general is capable to generate significantly higher amounts of C18 based SL. This can be observed in the three exceptional mutants, upon CerS inhibition with FB1 (see Figure 5-5) and after stimulation with increased amounts of L-serine (see Figure 5-15). The capability to produce increased amounts of sphingoid bases explains the necessity of additional regulatory mechanisms for the *de novo* synthesis (e.g. the SPOTS complex described in yeast) to keep the sphingolipid levels constant. Obviously this regulation is quite tight and still functional even in our cell culture model. Although not too much is known about the regulation of SPT activity in mammalian cells, regulatory elements like ORMs and ssSPTs might play a key role in the regulation of the sphingolipids rheostat by direct interaction with SPT.

The presence of the CerS inhibitor FB1 obviously disturbs this regulatory mechanism by blocking the formation of downstream products of SPT. The presence of FB1 provokes the formation of 1-deoxySL even in wild type SPT (29). Diverse additional side effects of this inhibitor besides the observed accumulation of free bases were reported (40). In our model, the addition of FB1 caused significant differences in the canonical activity between the mutants (see Figure 5-5). While sphingolipid *de novo* synthesis in general seems to be stimulated by the inhibition of CerS (e.g. SPTLC2 increased from 340 to 750pmol/mil cells and 24 hours), several mutants, including the hyperactive SPTLC1 p.S331F, S331Y and SPTLC1 p. I504F, showed clearly reduced activity in comparison to the wt expressing cells. The general pattern for 1-deoxySL formation however was not altered by FB1 (see Figure 5-7). However, the total amount of 1-deoxySL formed were up to ten fold higher than in the absence of FB1. This observations goes along with the above mentioned stimulatory effect of FB1 on 1-deoxySL formation by SPT (29).

The activities measured in the presence of fumonisin B1 are closer to the maximal activity of SPT, as it is measured by the *in vitro* enzyme assays, however they do not reflect a physiological state. At present we do not know enough about the underlying mechanisms causing the selective induction of 1-deoxySL synthesis. Therefore, these data should to be interpreted carefully and were also not considered as reliable discriminators for the clustering of the mutations.

Three out of the 17 analyzed mutations seem to escape the proposed feedback regulation of the SL *de novo* synthesis and showed a significantly increased canonical SPT activity compared to the wt enzyme. SPTLC1 p.S331F, p.S331Y and SPTLC2 p.I504F are confirmed and non-inherited *de novo* mutations identified in five non-related HSAN1 patients (3 x p.S331F, 1 x p.S331Y and 1 x p.I504F).

Interestingly orthologs of these three mutations were found in a yeast screen to compensate for the loss of SPT activation by the small protein TSC3.

In 2002 Monaghan reported the yeast SPT mutation LCB1 p.I491F (the ortholog of SPTLC2 p.I504F) to eliminate the dependency on additional activation by the ssSPT ortholog TSC3 for growth at restrictive temperature 37°C (52).

Seven years later Han and colleagues showed that the small subunits ssSPTa and ssSPTb not only affect SPTs activity but also its acyl CoA preference (51). The activation of SPT by the small subunits depended on the SPTLC2 mutation expressed. While SPTLC2 wild type, p.V359M and p.G382V were fully activated in the complex with SPTLC1 and ssSPTa, this was not the case for p.I504F which instead responded much better to additional activation by ssSPTb.

Recently Harmon et al expressed the human SPTLC1 p.S331F mutation together with SPTLC2 in a yeast SPT knockout strain and observed an altered interaction of the mutant with the small SPT subunits (53). SPT knockout cells co-expressing SPTLC1 p.S331F and SPTLC2 were able to grow without additional activation by ssSPTa while cells expressing SPTLC1 wild type needed this additional activation by ssSPTa to rescue the knockout phenotype. Furthermore the group showed that coexpression of SPTLC1 p. S331F and SPTLC2 together with ssSPTb but not ssSPTa resulted in additional activation (53).

Hence, mutations at the two loci (SPTLC1 p.S331 and SPTLC2 p.I504) obviously affect the interaction with ssSPTa/b in higher eukaryotes or TSC3 in yeast respectively. Harmon even identified a single residues (ssSPTa-M25) which plays a crucial role in the interaction of ssSPTa and SPTLC1 p.S331F and is responsible for the reduced activation due to impaired interaction of both proteins. In ssSPTb the residues methionine 25 is replaced by valine which does not cause any steric interference with the mutated SPTLC1 p.S331F and therefore interaction and activation are still possible.

These observations together with our findings of a highly increased canonical activity and increased formation of C20 SL support the hypothesis that a mutation in the residues SPTLC1 S331 and SPTLC2 I504 impairs the regulation of SPT and de-novo sphingolipid

synthesis. This deregulation is most likely caused by an impaired and changed interaction with the small subunits ssSPTa or ssSPTb respectively.

Mutations at both residues (SPTLC1 p.S331 and SPTLC2 p.I504) render the mutated enzyme independent on additional activation by the regulatory small subunits, explaining the increased basal activity with the canonical substrate L-serine, which we observed in our assays. The additional finding that the mutations p.S331F and p.I504F are activated by ssSPTb rather than ssSPTa together with the fact that overexpression of ssSPTb changes SPT's substrate preference towards stearoyl-CoA goes along with our observation of an increased formation of C20-sphingoid backbones. The pathology of these patients is clearly distinct from other HSAN1 cases. It is characterized by an early or even congenital onset of symptoms, severe muscular impairment, growth retardation, early development of cataracts and impairment of the respiratory system (20, 21, 25, 35). Correlation between the unique biochemical properties in cell culture, alterations of the sphingolipids profiles in plasma of the affected patient and a clinically severe phenotype was obvious in those three cases. Comparison of the biochemistry of p.S331F, p.S331Y and p.I504F to other HSAN1 mutations (e.g. p.C133W) showed the most striking differences in an increased canonical activity and the increased preference for C18-acyl-CoA. Therefore we assume that those two factors might be related to the clinically more severe symptoms in these patients. However, the exact mode of action for these atypical and minor sphingoid bases in the disease pathology still remains to be determined.

The presence of elevated atypical 1-deoxySL in plasma is considered a hallmark of HSAN1.

The typical and most frequently reported HSAN1 mutations SPTLC1 p.C133W, p.C133Y and also the recently found SPTLC2 mutations p.A182P, p.G382V and p.S384F are clearly associated with HSAN1 and show an increased formation of 1-deoxysphingoid bases in our cell culture assays as well. Furthermore the total concentration of 1-deoxysphingoid bases in plasma of p.C133W patients was shown to correlate with the severity of the phenotype in these patients (54).

However some benign or non-disease associated mutations in SPT like SPTLC1 p.R151L, p.G382V and several other mutations from the first cluster cause neither increased activity with L-Alanine nor a reduced canonical activity. Mutations like SPTLC1 p.V144D, the p.T409M and the recently found p.A339V (see chapter 4 of this thesis) fail to fit into the expected picture as well.

Allthough the p.V144D mutation is one of the first annotated HSAN1 mutations in SPTLC1 it had no significant influence on substrate preference and activity of the holoenzyme in our model and failed to increase incorporation of alanine in the isotope labelling assay. Therefore it was grouped together with p.A339V and p.T409M into the first cluster. Nevertheless patients carrying any of these mutations present with elevated 1-deoxySL plasma levels and a HSAN1 phenotype. And for p.A339V we even showed, that the mutation confers the capability to increase the 1-deoxySL formation at least upon L-alanine stimulation (see chapter 4 of this thesis).

With the first reports on regulatory elements affecting SPT's activity and substrate preference the whole *de novo* generation of sphingolipids became more complex. Although in 2001 the cause of HSAN1 was originally mapped to SPT, we cannot exclude that other mutations outside SPT impair its regulation or maybe the degradation of its typical and atypical products. More research on these branches of sphingolipids metabolism is absolutely necessary to unravel potential alternative mechanisms causing elevated 1-deoxySL concentrations.

Together with the here included SPTLC1 p.A339V in total three new mutations in SPTLC1 were found and reported in the context of HSAN1 within one year. This highlights the genetic heterogenity of this disease but also the need of ongoing research to unravel the various underlying mechanisms, causing the death of sensory peripheral neurons.

5.6 References for chapter 5:

1. Dyck, P. J. (1993) Neuronal atrophy and degeneration predominantly affecting peripheral sensory and autonomic neurons. in *Peripheral Neuropathy*, 3rd Editio (Dyck, P. J., Thomas, K. P., Griffin, J. W., Low, P. A., and Poduslo, J. F. eds), pp. 1065–1093, Saunders Philladelphia

2. Leipold, E., Liebmann, L., Korenke, G. C., Heinrich, T., Giesselmann, S., Baets, J., Ebbinghaus, M., Goral, R. O., Stödberg, T., Hennings, J. C., Bergmann, M., Altmüller, J., Thiele, H., Wetzel, A., Nürnberg, P., Timmerman, V., De Jonghe, P., Blum, R., Schaible, H.-G., Weis, J., Heinemann, S. H., Hübner, C. a, and Kurth, I. (2013) A *de novo* gain-of-function mutation in SCN11A causes loss of pain perception. *Nat. Genet.* 45, 1399–404

3. Edvardson, S., Cinnamon, Y., Jalas, C., Shaag, A., Maayan, C., Axelrod, F. B., and Elpeleg, O. (2012) Hereditary sensory autonomic neuropathy caused by a mutation in dystonin. *Ann. Neurol.* 71, 569–72

4. Auer-Grumbach, M. (2008) Hereditary sensory neuropathy type I. *Orphanet J. Rare Dis.* 3, 7

5. Houlden, H., King, R., Blake, J., and Groves, M. (2006) Clinical, pathological and genetic characterization of hereditary sensory and autonomic neuropathy type 1 (HSAN I). *Brain*. 1, 411–425

6. Denny-Brown, D. (1951) Hereditary sensory radicular neuropathy. *J. Neurol. Neurosurg. Psychiatry*. [online] http://jnnp.bmj.com/content/14/4/237 (Accessed September 3, 2012)

7. Rotthier, A., Baets, J., Timmerman, V., and Janssens, K. (2012) Mechanisms of disease in hereditary sensory and autonomic neuropathies. *Nat. Rev. Neurol.* 8, 73–85

8. Hanada, K. (2003) Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids*. 1632, 16–30

9. Hornemann, T., Richard, S., Rütti, M. F., Wei, Y., von Eckardstein, A., Serine-palmitoyltransferase, M., Ru, M. F., and Eckardstein, A. Von (2006) Cloning and initial characterization of a new subunit for mammalian serine-palmitoyltransferase. *J. Biol. Chem.* 281, 37275–81

10. Hornemann, T., Penno, A., Rütti, M. F., Ernst, D., Kivrak-Pfiffner, F., Rohrer, L., and von Eckardstein, A. (2009) The SPTLC3 subunit of serine palmitoyltransferase generates short chain sphingoid bases. *J. Biol. Chem.* 284, 26322–30

11. Hornemann, T., Wei, Y., and Eckardstein, A. V. O. N. (2007) Is the mammalian serine palmitoyltransferase a high-molecular-mass complex ? 164, 157–164

12. Rautenstrauss, B., Neitzel, B., Muench, C., Haas, J., and Holinski-Feder, E. (2009) LATE ONSET HEREDITARY SENSORY NEUROPATHY TYPE 1 (HSN1) CAUSED BY A NOVEL P.C133R MISSENSE MUTATION IN SPTLC1 Würzburg, Germany. in *2009 Meeting of the Peripheral Nerve Society July 4-8, 2009*, p. 290 of 381

13. Bejaoui, K., Wu, C., Scheffler, M. D., Haan, G., Ashby, P., Wu, L., de Jong, P., and Brown, R. H. (2001) SPTLC1 is mutated in hereditary sensory neuropathy, type 1. *Nat. Genet.* 27, 261–2

14. Dawkins, J. L., Hulme, D. J., Brahmbhatt, S. B., Auer-Grumbach, M., and Nicholson, G. a (2001) Mutations in SPTLC1, encoding serine palmitoyltransferase, long chain base subunit-1, cause hereditary sensory neuropathy type I. *Nat. Genet.* 27, 309–12

15. Meggouh, F., Bienfait, H. M. E., Weterman, M. a J., de Visser, M., and Baas, F. (2006) Charcot-Marie-Tooth disease due to a *de novo* mutation of the RAB7 gene. *Neurology*. 67, 1476–8

16. Sjöblom, T., Jones, S., Wood, L. D., Parsons, D. W., Lin, J., Barber, T. D., Mandelker, D., Leary, R. J., Ptak, J., Silliman, N., Szabo, S., Buckhaults, P., Farrell, C., Meeh, P., Markowitz, S. D., Willis, J., Dawson, D., Willson, J. K. V, Gazdar, A. F., Hartigan, J., Wu, L., Liu, C., Parmigiani, G., Park, B. H., Bachman, K. E., Papadopoulos, N., Vogelstein, B., Kinzler, K. W., and Velculescu, V. E. (2006) The consensus coding sequences of human breast and colorectal cancers. *Science*. 314, 268–74

17. Hanada, K. (1998) Mammalian Cell Mutants Resistant to a Sphingomyelin-directed Cytolysin. GENETIC AND BIOCHEMICAL EVIDENCE FOR COMPLEX FORMATION OF THE LCB1 PROTEIN WITH THE LCB2 PROTEIN FOR SERINE PALMITOYLTRANSFERASE. *J. Biol. Chem.* 273, 33787–33794

18. Momin, A. a, Park, H., Allegood, J. C., Leipelt, M., Kelly, S. L., Merrill, A. H., and Hanada, K. (2009) Characterization of mutant serine palmitoyltransferase 1 in LY-B cells. *Lipids*. 44, 725–32

19. Davidson, G. L., Murphy, S. M., Polke, J. M., Laura, M., Salih, M. A. M., Muntoni, F., Blake, J., Brandner, S., Davies, N., Horvath, R., Price, S., Donaghy, M., Roberts, M., Foulds, N., Ramdharry, G., Soler, D., Lunn, M. P., Manji, H., Davis, M. B., Houlden, H., and Reilly, M. M. (2012) Frequency of mutations in the genes associated with hereditary sensory and autonomic neuropathy in a UK cohort. *J. Neurol.* 259, 1673–1685

20. Rotthier, A., Baets, J., De Vriendt, E., Jacobs, A., Auer-Grumbach, M., Lévy, N., Bonello-Palot, N., Kilic, S. S., Weis, J., Nascimento, A., Swinkels, M., Kruyt, M. C., Jordanova, A., De Jonghe, P., and Timmerman, V. (2009) Genes for hereditary sensory and autonomic neuropathies: a genotype-phenotype correlation. *Brain*. 132, 2699–711

21. Auer-Grumbach, M., Bode, H., Pieber, T. R., Schabhüttl, M., Fischer, D., Seidl, R., Graf, E., Wieland, T., Schuh, R., Vacariu, G., Grill, F., Timmerman, V., Strom, T. M., and Hornemann, T. (2013) Mutations at Ser331 in the HSN type I gene SPTLC1 are associated with a distinct syndromic phenotype. *Eur. J. Med. Genet.* 56, 266–9

22. Verhoeven, K., Coen, K., De Vriendt, E., Jacobs, A., Van Gerwen, V., Smouts, I., Pou-Serradell, A., Martin, J.-J., Timmerman, V., and De Jonghe, P. (2004) SPTLC1 mutation in twin sisters with hereditary sensory neuropathy type I. *Neurology*. 62, 1001–1002

23. Hornemann, T., Penno, A., Richard, S., Nicholson, G., van Dijk, F. S., Rotthier, A., Timmerman, V., and von Eckardstein, A. (2009) A systematic comparison of all mutations in

hereditary sensory neuropathy type I (HSAN I) reveals that the G387A mutation is not disease associated. *Neurogenetics*. 10, 135–43

24. Murphy, S. M., Ernst, D., Wei, Y., Laurà, M., Liu, Y.-T., Polke, J., Blake, J., Winer, J., Houlden, H., Hornemann, T., and Reilly, M. M. (2013) Hereditary sensory and autonomic neuropathy type 1 (HSANI) caused by a novel mutation in SPTLC2. *Neurology*. 80, 2106–11

25. Rotthier, A., Auer-Grumbach, M., Janssens, K., Baets, J., Penno, A., Almeida-Souza, L., Van Hoof, K., Jacobs, A., De Vriendt, E., Schlotter-Weigel, B., Löscher, W., Vondráček, P., Seeman, P., De Jonghe, P., Van Dijck, P., Jordanova, A., Hornemann, T., and Timmerman, V. (2010) Mutations in the SPTLC2 subunit of serine palmitoyltransferase cause hereditary sensory and autonomic neuropathy type I. *Am. J. Hum. Genet.* 87, 513–22

26. Ernst, D. (2013) Regulation of Deoxy-Sphingolipids and Their Role in Disease. *PhD Thesis*

27. Ernst, D., Murphy, S. M., Sathiyanadan, K., Wei, Y., Othman, A., Laura, M., Donaghy, M., Houlden, H., Reilly, M. M., and Hornemann, T. (2015) Novel HSAN1 Mutation in Serine Palmitoyltransferase Resides at a Putative Phosphorylation Site That Is Involved in Regulating Substrate Specificity. *NeuroMolecular Med.* 17, 47–57

28. Penno, A., Reilly, M. M., Houlden, H., Laurá, M., Rentsch, K., Niederkofler, V., Stoeckli, E. T., Nicholson, G., Eichler, F., Brown, R. H., von Eckardstein, A., and Hornemann, T. (2010) Hereditary sensory neuropathy type 1 is caused by the accumulation of two neurotoxic sphingolipids. *J. Biol. Chem.* 285, 11178–87

29. Zitomer, N. C., Mitchell, T., Voss, K. a, Bondy, G. S., Pruett, S. T., Garnier-Amblard, E. C., Liebeskind, L. S., Park, H., Wang, E., Sullards, M. C., Merrill, A. H., and Riley, R. T. (2009) Ceramide synthase inhibition by fumonisin B1 causes accumulation of 1-deoxysphinganine: a novel category of bioactive 1-deoxysphingoid bases and 1-deoxydihydroceramides biosynthesized by mammalian cell lines and animals. *J. Biol. Chem.* 284, 4786–95

30. Cuadros, R., Montejo de Garcini, E., Wandosell, F., Faircloth, G., Fernández-Sousa, J. M., and Avila, J. (2000) The marine compound spisulosine, an inhibitor of cell proliferation, promotes the disassembly of actin stress fibers. *Cancer Lett.* 152, 23–9

31. Baird, R. D., Kitzen, J., Clarke, P. a, Planting, A., Reade, S., Reid, A., Welsh, L., López Lázaro, L., de las Heras, B., Judson, I. R., Kaye, S. B., Eskens, F., Workman, P., deBono, J. S., and Verweij, J. (2009) Phase I safety, pharmacokinetic, and pharmacogenomic trial of ES-285, a novel marine cytotoxic agent, administered to adult patients with advanced solid tumors. *Mol. Cancer Ther.* 8, 1430–7

32. Schöffski, P., Dumez, H., Ruijter, R., Miguel-Lillo, B., Soto-Matos, a, Alfaro, V., and Giaccone, G. (2011) Spisulosine (ES-285) given as a weekly three-hour intravenous infusion: results of a phase I dose-escalating study in patients with advanced solid malignancies. *Cancer Chemother. Pharmacol.* 68, 1397–403

33. Huehne, K., Zweier, C., Raab, K., Odent, S., Bonnaure-Mallet, M., Sixou, J.-L., Landrieu, P., Goizet, C., Sarlangue, J., Baumann, M., Eggermann, T., Rauch, A., Ruppert, S., Stettner, G. M., and Rautenstrauss, B. (2008) Novel missense, insertion and deletion

mutations in the neurotrophic tyrosine kinase receptor type 1 gene (NTRK1) associated with congenital insensitivity to pain with anhidrosis. *Neuromuscul. Disord.* 18, 159–66

34. Rotthier, A., Penno, A., Rautenstrauss, B., Auer-Grumbach, M., Stettner, G. M., Asselbergh, B., Van Hoof, K., Sticht, H., Lévy, N., Timmerman, V., Hornemann, T., and Janssens, K. (2011) Characterization of two mutations in the SPTLC1 subunit of serine palmitoyltransferase associated with hereditary sensory and autonomic neuropathy type I. *Hum. Mutat.* 32, E2211–25

35. Suh, B. C., Hong, Y. Bin, Nakhro, K., Nam, S. H., Chung, K. W., and Choi, B.-O. (2014) Early-onset severe hereditary sensory and autonomic neuropathy type 1 with S331F SPTLC1 mutation. *Mol. Med. Rep.* 9, 481–6

36. Riley, R. T., Norred, W. P., Wang, E., and Merrill, a H. (1999) Alteration in sphingolipid metabolism: bioassays for fumonisin- and ISP-I-like activity in tissues, cells and other matrices. *Nat. Toxins*. 7, 407–14

37. Bejaoui, K., Uchida, Y., Yasuda, S., Ho, M., Nishijima, M., Brown, R. H., Holleran, W. M., and Hanada, K. (2002) Hereditary sensory neuropathy type 1 mutations confer dominant negative effects on serine palmitoyltransferase, critical for sphingolipid synthesis. *J. Clin. Invest.* 110, 1301–8

38. McCampbell, A., Truong, D., Broom, D. C., Allchorne, A., Gable, K., Cutler, R. G., Mattson, M. P., Woolf, C. J., Frosch, M. P., Harmon, J. M., Dunn, T. M., and Brown, R. H. (2005) Mutant SPTLC1 dominantly inhibits serine palmitoyltransferase activity in vivo and confers an age-dependent neuropathy. *Hum. Mol. Genet.* 14, 3507–21

39. Gable, K., Han, G., Monaghan, E., Bacikova, D., Natarajan, M., Williams, R., and Dunn, T. M. (2002) Mutations in the yeast LCB1 and LCB2 genes, including those corresponding to the hereditary sensory neuropathy type I mutations, dominantly inactivate serine palmitoyltransferase. *J. Biol. Chem.* 277, 10194–200

40. Riley, R. T., Wang, E., Schroeder, J. J., Smith, E. R., Plattner, R. D., Abbas, H., Yoo, H. S., and Merrill, a H. (1996) Evidence for disruption of sphingolipid metabolism as a contributing factor in the toxicity and carcinogenicity of fumonisins. *Nat. Toxins*. 4, 3–15

41. Garofalo, K., Penno, A., and Schmidt, B. (2011) Oral l-serine supplementation reduces production of neurotoxic deoxysphingolipids in mice and humans with hereditary sensory autonomic neuropathy type 1. *J. Clin. …*. 10.1172/JCI57549.)

42. Merrill, A. H. (1983) Characterization of serine palmitoyltransferase activity in Chinese hamster ovary cells. *Biochim. Biophys. Acta*. 754, 284–91

43. Williams, D., and Merrill, H. (1984) Enzymology of Long-Chain Base Synthesis by Liver : Characterization Serine Palmitoyltransferase in Rat Liver Microsomes. *Arch. Biochem. Biophys.* 228, 282–291

44. Dedov, V. N., Dedova, I. V, Merrill, A. H., and Nicholson, G. a (2004) Activity of partially inhibited serine palmitoyltransferase is sufficient for normal sphingolipid metabolism and viability of HSN1 patient cells. *Biochim. Biophys. Acta*. 1688, 168–75

45. Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., and Merrill, A. H. (1991) Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with Fusarium moniliforme. *J. Biol. Chem.* 266, 14486–90

46. Yoo, H. S., Norred, W. P., Wang, E., Merrill, a H., and Riley, R. T. (1992) Fumonisin inhibition of *de novo* sphingolipid biosynthesis and cytotoxicity are correlated in LLC-PK1 cells. *Toxicol. Appl. Pharmacol.* 114, 9–15

47. Breslow, D. K., Collins, S. R., Bodenmiller, B., Aebersold, R., Simons, K., Shevchenko, A., Ejsing, C. S., and Weissman, J. S. (2010) Orm family proteins mediate sphingolipid homeostasis. *Nature*. 463, 1048–53

48. Han, S., Lone, M. A., Schneiter, R., and Chang, A. (2010) Orm1 and Orm2 are conserved endoplasmic reticulum membrane proteins regulating lipid homeostasis and protein quality control. *Proc. Natl. Acad. Sci. U. S. A.* 107, 5851–6

49. Roelants, F. M., Breslow, D. K., Muir, A., Weissman, J. S., and Thorner, J. (2011) Protein kinase Ypk1 phosphorylates regulatory proteins Orm1 and Orm2 to control sphingolipid homeostasis in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.* 108, 19222–7

50. Hjelmqvist, L., Tuson, M., Marfany, G., Herrero, E., Balcells, S., and Gonzàlez-Duarte, R. (2002) ORMDL proteins are a conserved new family of endoplasmic reticulum membrane proteins. *Genome Biol.* 3, RESEARCH0027

51. Han, G., Gupta, S. D., Gable, K., Niranjanakumari, S., Moitra, P., Eichler, F., Brown, R. H., Harmon, J. M., and Dunn, T. M. (2009) Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities. *Proc. Natl. Acad. Sci. U. S. A.* 106, 8186–91

52. Monaghan, E., Gable, K., and Dunn, T. (2002) Mutations in the Lcb2p subunit of serine palmitoyltransferase eliminate the requirement for the TSC3 gene in Saccharomyces cerevisiae. *Yeast*. 19, 659–70

53. Harmon, J. M., Bacikova, D., Gable, K., Gupta, S. D., Han, G., Sengupta, N., Somashekarappa, N., and Dunn, T. M. (2013) Topological and functional characterization of the ssSPTs, small activating subunits of serine palmitoyltransferase. *J. Biol. Chem.* 288, 10144–53

54. Laurá, M., Murphy, S. M., Hornemann, T., Bode, H., Polke, J., Blake, J., Houlden, H., and Reilly, M. M. (2012) P42 Hereditary sensory neuropathy type 1: correlation of severity and plasma atypical deoxy-sphyngoid bases. *Neuromuscul. Disord.* 22, S18

General discussion:

In this work we compared the biochemical characteristics of all HSAN1-associated mutations in SPT. We reported two new mutations in SPTLC1 (p.S331Y and p.A339V), that were isolated from HSAN1 patients, which despite the close proximity of the mutations presented very distinct clinical phenotypes. We obtained new insights from therapeutic trials and new diagnostic approaches which are of importance for treatment and diagnosis of HSAN1 and maybe even peripheral neuropathies in general.

We developed and optimized a set of assays that allows for the fast and reproducible determination of the most interesting biochemical parameters of SPT. The use of deuterated substrates followed by mass spectrometric analysis allows us to measure the intracellular activity and product profile of the SPT complex under virtually any desired condition.

In 1996 Riley already reviewed the impacts and side effects of the disruption of sphingolipid metabolism by fumonisins (1). Fumonisins in general and specifically fumonisin B1 exert a long list of direct and indirect effects on various cell lines, organs, and whole organisms. The list for Swiss 3T3 fibroblasts includes growth inhibition and apoptosis in cultured cells, increased permeability of aortic endothelial cells, increased DNA synthesis and reduced secretion of complex sphingolipids, bound to lipoproteins from the liver, just to mention a few (1). The sheer number of wanted and unwanted effects of FB1 on individual cells or whole organisms therefore might explain the usually encountered difficulties to interpret results obtained from FB1-treated cells. Nevertheless, the use of FB1 has become one of the standard methods in the field for the investigation of SPT activity.

Our metabolic labeling approach however has proven to offer the same, if not even better selectivity, as the classical FB1 assay for 1-deoxySL analysis described by Zitomer and colleagues (2). Furthermore, our approach allows for the determination of SPT activity in more physiological conditions.

The results obtained under FB1 treatment are still valid measurements. However, the underlying mechanisms that led to these extraordinarily high values in some cases (e.g. SPTLC2 p.A182P) need to be further studied and understood prior to drawing final conclusions from them. The metabolic labeling approach reduces this complexity, as it does not interfere with any enzyme of the sphingolipid synthesis pathway.

We showed that the exchange of serine at position 331 of SPTLC1 to phenylalanine or tyrosine causes a severe phenotype with additional or more severe symptoms such as early onset, severe muscle wasting and hypotonia, growth retardation, anhydrosis, respiratory

complications and formation of cataracts, which are not caused by other mutations in SPTLC1.

Another mutation in SPTLC2 (p.I504F) also causes a debilitating and severe phenotype (4). This mutation presents comparable biochemical characteristics to p.S331F/Y, with increased canonical activity and increased use of alternative amino acids but also acyl-CoAs. Based on our observations we state a clear correlation between these three genotypes and their associated severe phenotypes. Although the underlying mechanism is still largely unknown, several hints from experiments in yeast strongly argue for a disturbed interaction of the mutated subunits with the regulatory subunits ssSPTa and ssSPTb as a cause for this atypical activity pattern of SPT in cells and patients bearing one of these mutations (5–7). Unlike in yeast, the mammalian SPT seems not to depend on the presence and functionality of the ssSPTs to fulfill its canonical function. However, co-expression of the small subunits together with SPTLC1 and SPTLC2 alters the acyl-CoA preference of the SPT complex (8). Altered acyl-CoA preference together with increased activities with L-serine and L-alanine are the specific hallmarks of these three mutations and seem to be crucially involved in the development of the associated severe phenotypes.

More work is needed to investigate the complex regulation of sphingolipid *de novo* synthesis and the roles of all reported SPT-binding proteins (e.g. ssSPTa and b, ORMDL1-3 and Sac1).

For patients, the presence of elevated levels of C20 SLs can be used as an additional differentiator to discriminate the described rare genotypes (SPTLC1 p.S331F/Y and SPTLC2 p.I504F) from other HSAN1 genotypes. Phenotypical presence of anhydrosis, impaired vision, and respiratory complications allow a simple distinction of the three severe phenotypes from typical HSAN1. The atypical severe symptoms on the other hand might hamper the correct diagnosis, as early onset and anhydrosis are also typical symptoms of congenital insensitivity to pain with anhydrosis (CIPA or HSAN4). Misdiagnosis of patients with peripheral neuropathies is a common problem and confusion of severe HSAN1 with HSAN4 may have occurred on more than one occasion (10).

This again highlights the heterogeneity of HSAN and why profound knowledge of phenotypes without identification of the causative genotype cannot guarantee a correct diagnosis.

Proper classification of patients recently became even more important, as with the launch of the serine therapy, a promising real treatment for HSAN1 patients bearing a mutation in SPT became available. We showed that oral L-serine supplementation lowers the 1-deoxySL levels in mice expressing the SPTLC1 p.C133W mutation, and even patients bearing the p.C133Y mutation (11). Following up these initial results, a double blinded long-term study on

the positive effects of L-serine was launched. Preliminary results showed that the 1-deoxySL concentrations in the plasma of several participants bearing the SPTLC1 p.C133Y variant were again significantly lowered and their overall condition improved (data not shown).

Here we also reported a new experimental L-serine trial with a patient that was initially diagnosed with HSAN4 and was later shown to bear the rare SPTLC1 p.S331F mutation (10, 12). The p.S331F genotype of the patient is associated with an exceptionally severe phenotype. With our *in vitro* model, we showed that besides increased substrate promiscuity (concerning both the amino acid but also the acyl-CoA), this special mutation also causes an increased canonical SPT activity. Nevertheless, we did not measure increased concentrations of the normal C18-based sphingolipids in the plasma of our patients during a follow-up of almost two years. The 1-deoxySL concentrations in the plasma of the patient however were significantly lowered to about 50% of the baseline levels under L-serine supplementation. Additionally the patient started to gain weight and reported improved hair growth, wound healing and skin robustness. No adverse effects that could be assigned to the L-serine supplementation were observed in either this severely affected p.S331F patient nor in the p.C133Y-patients. Serine *de novo* synthesis was found to be activated by several types of cancer and feeding of rats with a serine and glycine free diet highly reduced tumor growth (for a recent review of L-serine´s role in cancer see (13)). This suggests a potentially increased risk of cancer development and faster tumor growth for serine supplemented patients. None of the current patients under L-serine treatment recently developed a tumor, but the therapy is also too new to exclude long-term effects.

Even with the artificially elevated high abundance of free serine in the blood, the plasma concentrations of the canonical SPT products do not change significantly. This shows again the complexity and tight regulation of sphingolipid *de novo* synthesis and the need for further investigations to understand the function of this rheostat.

Recently, elevated plasma levels of 1-deoxySLs were identified as novel biomarkers for the metabolic syndrome and type 2 diabetes (14). A follow-up study with streptozotocin-induced type 1 diabetes in rats revealed significantly lower 1-deoxySL levels in the rats which were fed with an L-serine-enriched diet. Mechanical sensitivity and nerve conduction velocities were improved (15), and even the development of cataracts was prevented in the L-serine supplemented rats (personal communication). These findings indicate promising beneficial effects of the L-serine therapy also for the phenotypically similar diabetic polyneuropathy.

Further extension of the serine therapy to other types of peripheral neuropathy with higher prevalence (e.g. HSAN2 and 4 and also diabetic polyneuropathy) might provide some surprising positive results.

Besides the identification of the three mutations resulting in the severe phenotype forming this special cluster within the HSAN1-associated mutations of SPT, the analysis of the other mutations confirmed our previous observations. We found that five more mutations cause an increased activity with L-alanine and all of them also cause at least the typical phenotype of HSAN1 with onset between 2nd and 5th decade, sensory loss, and development of ulcerations predominantly at the feet. The p.A182P mutation of SPTLC2 displayed unique characteristics, as it was associated with an earlier disease onset in the first decade of life and prominent motor involvement (16). Furthermore it presented the highest activity with L-alanine under physiological conditions, and even more increased activities under FB1 conditions. The canonical activity was not increased in normal cell culture conditions and even reduced under FB1 treatment. This mutation seemed to support specifically the formation of 1-deoxySLs, while the canonical activity of SPT was not affected. Further investigations of the structural changes and altered interaction patterns caused by this special mutation at Ala182 might help to finally understand the underlying structural changes in SPT that foster the formation of 1-deoxySL formation.

However, several other mutations failed to increase 1-deoxySL formation in our *in vitro* assays, but patients bearing one of these mutations (e.g. p.V144D or p.R151L) nevertheless present with significantly elevated concentrations of 1-deoxySLs in their plasma and variable symptoms of peripheral neuropathy.

This gives rise to questions which need to be addressed in the future. It is still unclear how far the levels of 1-deoxySLs in plasma reflect the situation in the affected nerves. Also, the origin of plasma 1-deoxySLs in the patients is still unclear. The majority of 1-deoxySLs in healthy individuals is suggested to be generated in the liver. In diabetes patients this could be explained by increased concentrations of L-alanine in this organ (17).

We also reported another interesting mutation (SPTLC1 p.A339V). The index patient and three more members of his family present a mild but typical phenotype of HSAN1 with late onset, loss of sensation in distal limbs, shooting pains, and impaired wound healing. However, the identified p.A339V mutation in SPTLC1 does not cause increased activity with alanine, nor does it reduce the canonical activity of SPT under physiological conditions. Challenged with a high dose of L-alanine however, the mutation caused a significant reduction of canonical activity and a significant increase of alternative activity of SPT. Furthermore, as for several other mutations, all affected individuals of this kinship showed significantly elevated 1-deoxySL concentrations in their plasma. The pedigree of the family nevertheless strongly argued for a not disease-causing benign mutation, as one son and the brother of the index case did not have the genotype and one more female carriers did not show the phenotype.

The p.A339V mutation actually can induce 1-deoxySL formation within cultured cells and those markers were found in all affected individuals. The origin of these atypical sphingolipids in the two non-carriers needs to be further investigated.

For several years mutations in SPT were the only known source for elevated concentrations of 1-deoxySLs. Only recently, increased levels of 1-deoxySLs were also reported in the context of type 2 diabetes and metabolic syndrome (14, 18). The two non-carriers out of our p.A339V family therefore are not the only cases with an unclear source for the elevated 1-deoxySL concentrations in their plasma. Additional mutations in yet unsuspicious genes involved in amino acid metabolism and transport or regulation of SPT activity might finally explain this phenomenon, but these still need to be unraveled.

Comparing all known and conclusively associated mutations showed that both locus and the type of mutation determine the catalytic activity and substrate preferences of the formed mutant SPT complex. These biochemical properties furthermore also represent crucial factors for the severity of the clinical phenotype of the patients.

In conclusion, we confirmed that the genotypic variability of HSAN1A and C is also reflected in a pronounced phenotypic variability. Nevertheless the different mutations can be classified based on their biochemical characteristics. The herein introduced new diagnostic and therapeutic approaches will help to further improve the understanding of peripheral neuropathies such as HSAN1.

5.7 Outlook on potential future experiments

Our metabolic labeling approach allows the addition of new mutants of SPT, or even other neuropathy-associated genes, quite easily to the here reported dataset. We will continue and also recommend adding as many mutations as possible to this overview, as this will help to build a bigger picture of genotypes and their associated biochemical phenotypes, which will finally contribute to a better understanding of HSAN1 and the underlying pathomechanism.

For neuropathy patients with suspected HSAN1, elevated concentrations of the atypical 1-deoxysphingolipids and the C20-based sphingolipids in plasma are a fast and cheap alternative to genotyping that allows for a definite diagnosis and conclusive identification of SPT as the culprit. Analysis of the full sphingolipid profile therefore should definitely be considered a valid diagnostic approach for HSAN1 suspected patients.

The *de novo* synthesis of sphingolipids and also 1-deoxySL is obviously highly regulated and we still do not know all regulatory elements in this pathway. We found strong hints for an impaired interaction of the mutated SPT with the known regulatory proteins ssSPTa and b.

Expression analysis for ssSPTa and ssSPTb in tissues such as a skin and nerve biopsies or even blood could be a first step to investigate an altered interaction patterns between the three deregulated mutants (SPTLC1 p.S331F/Y and SPTLC2 p.I504F) and the other known mutations of SPT.

Furthermore we showed that, mutations in SPT are not the only source for increased 1-deoxySL levels, and it is likely, that mutations in regulatory elements play an important role as well.

Whole exome sequencing of families like the p.A339V kinship would be a good next step towards the discovery of these additional factors supporting the increased substrate promiscuity of SPT. The potential presence of higher order mutations also implies the chance to finally understand how seventeen different genotypes can cause more or less only one phenotype.

Besides these genomic approaches more structural data on SPT and its interaction partners would be beneficial. Unfortunately the x-ray structure of the mammalian SPT is not available yet and all current models are based on the structure of the prokaryotic SPT, which was re-solved from two sphingolipid generating bacteria, *Sphingomonas paucimobilis* and *Sphingobacterium multivorum*. Those bacteria express soluble homodimeric forms of the enzyme, which are quite different from the mammalian protein.

Better structural data for mammalian SPT and its interaction partners therefore would help to understand the direct effects of the various mutations on the structure of the enzyme and also the impact of these changes on the interaction with regulatory proteins.

The currently available mouse model of HSAN1 is based on the systemic overexpression of SPTLC1 p.C133W on the wild type SPT background (9). The use of these mice therefore is limited. First of all it is limited to experiments on the typical phenotype of HSAN1. And furthermore it does not reflect the situation in the patients, which have one intact and one mutated allele of the respective subunit.

A more physiological model with a knock-in of the p.S331F or p.I504F mutation into one of the SPTLC1 alleles would be the preferable tool to study the processes, underlying these severe phenotypes, in more detail.

SPT is ubiquitously expressed and by now it is impossible to track the origin of the 1-deoxySL in the plasma from HSAN1 patients or the available mouse model.

A tissue specific knock-in of any HSAN1 mutant could solve this problem. Under control of a promotor of the peripheral nerve system (PNS) the formation of 1-deoxySL would increase only within the peripheral nerves. With PNS-specific expression, the majority of 1-deoxySL, measured in the plasma, must originate from the nerves and would reflect the situation in those much better.

Another important and question could be answered (at least partially) with this new model as well. It is not known, if the endogenous production or the uptake of sphingolipids and 1-deoxySL from the blood causes the intracellular accumulation of 1-deoxySL and the damage of the peripheral nerves. Tissue specific expression of the mutation should reduce the concentrations of 1-deoxySL in the plasma.

To further contain the lipids at the site of production, within the peripheral nerves, a cross breeding with another strain with impaired lipid transport (e.g. ABCA1 knockout) would be a good option to address this question.

Besides all these uncertainties and the need for a lot of additional work, there is also one very clear fact, which we confirmed in this thesis. The L-serine treatment of our p.S331F patient works fine and will be continued.

So far the serine therapy was not tested in patients with other forms of HSAN. The effectiveness of L-serine treatment in for example congenital insensitivity to pain with anhydrosis (CIPA or HSAN4) would be unexpected, as the genetic cause of HSAN4 (mutations in NTRK1) is neither involved in amino acid nor sphingolipid metabolism.

Therefore we recommend testing this therapy as potential treatment also for other neuropathies such as diabetic neuropathy or HSAN4. As 1-deoxySLs were not reported in HSAN4 patients, a significant improvement under therapy could answer the important question, whether the L-serine causes the beneficial, neurotrophic effects in a direct manner or via the lowering of the neurotoxic 1-deoxySL.

5.8 References for general discussion:

1. Riley, R. T., Wang, E., Schroeder, J. J., Smith, E. R., Plattner, R. D., Abbas, H., Yoo, H. S., and Merrill, a H. (1996) Evidence for disruption of sphingolipid metabolism as a contributing factor in the toxicity and carcinogenicity of fumonisins. *Nat. Toxins*. **4**, 3–15

2. Zitomer, N. C., Mitchell, T., Voss, K. a, Bondy, G. S., Pruett, S. T., Garnier-Amblard, E. C., Liebeskind, L. S., Park, H., Wang, E., Sullards, M. C., Merrill, A. H., and Riley, R. T. (2009) Ceramide synthase inhibition by fumonisin B1 causes accumulation of 1-deoxysphinganine: a novel category of bioactive 1-deoxysphingoid bases and 1-deoxydihydroceramides biosynthesized by mammalian cell lines and animals. *J. Biol. Chem.* **284**, 4786–95

3. Auer-Grumbach, M., Bode, H., Pieber, T. R., Schabhüttl, M., Fischer, D., Seidl, R., Graf, E., Wieland, T., Schuh, R., Vacariu, G., Grill, F., Timmerman, V., Strom, T. M., and Hornemann, T. (2013) Mutations at Ser331 in the HSN type I gene SPTLC1 are associated with a distinct syndromic phenotype. *Eur. J. Med. Genet.* **56**, 266–9

4. Rotthier, A., Auer-Grumbach, M., Janssens, K., Baets, J., Penno, A., Almeida-Souza, L., Van Hoof, K., Jacobs, A., De Vriendt, E., Schlotter-Weigel, B., Löscher, W., Vondráček, P., Seeman, P., De Jonghe, P., Van Dijck, P., Jordanova, A., Hornemann, T., and Timmerman, V. (2010) Mutations in the SPTLC2 subunit of serine palmitoyltransferase cause hereditary sensory and autonomic neuropathy type I. *Am. J. Hum. Genet.* **87**, 513–22

5. Monaghan, E., Gable, K., and Dunn, T. (2002) Mutations in the Lcb2p subunit of serine palmitoyltransferase eliminate the requirement for the TSC3 gene in Saccharomyces cerevisiae. *Yeast*. **19**, 659–70

6. Harmon, J. M., Bacikova, D., Gable, K., Gupta, S. D., Han, G., Sengupta, N., Somashekarappa, N., and Dunn, T. M. (2013) Topological and functional characterization of the ssSPTs, small activating subunits of serine palmitoyltransferase. *J. Biol. Chem.* **288**, 10144–53

7. Beattie, A. E., Gupta, S. D., Frankova, L., Harmon, J. M., Dunn, T. M., and Dominic, J. (2013) The PLP-dependent enzyme SPT ; effects of the small subunits and insights from bacterial mimics of human hLCB2a HSAN1 mutations . *Biomed Res. Int.* **2013**, 13

8. Han, G., Gupta, S. D., Gable, K., Niranjanakumari, S., Moitra, P., Eichler, F., Brown, R. H., Harmon, J. M., and Dunn, T. M. (2009) Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 8186–91

9. McCampbell, A., Truong, D., Broom, D. C., Allchorne, A., Gable, K., Cutler, R. G., Mattson, M. P., Woolf, C. J., Frosch, M. P., Harmon, J. M., Dunn, T. M., and Brown, R. H. (2005) Mutant SPTLC1 dominantly inhibits serine palmitoyltransferase activity in vivo and confers an age-dependent neuropathy. *Hum. Mol. Genet.* **14**, 3507–21

10. Huehne, K., Zweier, C., Raab, K., Odent, S., Bonnaure-Mallet, M., Sixou, J.-L., Landrieu, P., Goizet, C., Sarlangue, J., Baumann, M., Eggermann, T., Rauch, A., Ruppert, S., Stettner, G. M., and Rautenstrauss, B. (2008) Novel missense, insertion and deletion

mutations in the neurotrophic tyrosine kinase receptor type 1 gene (NTRK1) associated with congenital insensitivity to pain with anhidrosis. *Neuromuscul. Disord.* **18**, 159–66

11. Garofalo, K., Penno, A., and Schmidt, B. (2011) Oral l-serine supplementation reduces production of neurotoxic deoxysphingolipids in mice and humans with hereditary sensory autonomic neuropathy type 1. *J. Clin. …*. 10.1172/JCI57549.)

12. Rotthier, A., Penno, A., Rautenstrauss, B., Auer-Grumbach, M., Stettner, G. M., Asselbergh, B., Van Hoof, K., Sticht, H., Lévy, N., Timmerman, V., Hornemann, T., and Janssens, K. (2011) Characterization of two mutations in the SPTLC1 subunit of serine palmitoyltransferase associated with hereditary sensory and autonomic neuropathy type I. *Hum. Mutat.* **32**, E2211–25

13. Locasale, J. W. (2013) Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nat. Rev. Cancer*. **13**, 572–83

14. Othman, A., Rütti, M. F., Ernst, D., Saely, C. H., Rein, P., Drexel, H., Porretta-Serapiglia, C., Lauria, G., Bianchi, R., von Eckardstein, A., and Hornemann, T. (2012) Plasma deoxysphingolipids: a novel class of biomarkers for the metabolic syndrome? *Diabetologia*. **55**, 421–31

15. Othman, A., Bianchi, R., Alecu, I., Wei, Y., Porretta-Serapiglia, C., Lombardi, R., Chiorazzi, A., Meregalli, C., Oggioni, N., Cavaletti, G., Lauria, G., von Eckardstein, A., and Hornemann, T. (2015) Lowering Plasma 1-Deoxysphingolipids Improves Neuropathy in Diabetic Rats. *Diabetes*. **64**, 1035–1045

16. Murphy, S. M., Ernst, D., Wei, Y., Laurà, M., Liu, Y.-T., Polke, J., Blake, J., Winer, J., Houlden, H., Hornemann, T., and Reilly, M. M. (2013) Hereditary sensory and autonomic neuropathy type 1 (HSANI) caused by a novel mutation in SPTLC2. *Neurology*. **80**, 2106–11

17. Othman, A. M. A. (2013) Atypical Sphingolipids as Biomarkers and Therapeutic Targets in Cardio-metabolic Diseases. *PhD Thesis*

18. Bertea, M., Rütti, M. F., Othman, A., Marti-Jaun, J., Hersberger, M., von Eckardstein, A., and Hornemann, T. (2010) Deoxysphingoid bases as plasma markers in diabetes mellitus. *Lipids Health Dis.* **9**, 84